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14. ABSTRACT Chronic inflammation is now regarded as a promoting force for cancer development. We have previously demonstrated that inhibition of leukocyte migration and/or leukocyte-derived protease activities during squamous carcinogenesis significantly decrease tumor incidence; thus, supporting the contention that inflammation can be targeted pharmacologically to affect cancer outcome. Using the MMTV-PymT mouse model of mammary carcinogenesis, we now demonstrate that mammary carcinogenesis is similarly susceptible to immuno-modulation as genetic deletion of CD4+ T lymphocytes and/or a leukocyte cysteine protease (cathepsin C), significantly diminishes pulmonary metastasis formation. Utilizing a 3D organotypic culture system with primary cells, we have revealed that activated CD4+ T cells alter the mammary microenvironment in such a way that mammary epithelial cell migration into matrix is favored. Moreover, the paracrine mechanisms whereby T cells mediate these effects are in part due to M2-activation of macrophages in an IL-4-dependent manner. Together, these studies provide insight into the role adaptive immune cells play in regulating myeloid cell behavior and how leukocyte proteases together regulate cancer development, and will reveal novel mechanisms with which to target tumor cells with anti-cancer therapeutics and/or image inflammation associated with breast cancer development.

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I. INTRODUCTION:

The association of immune cells and cancer has been known for over a century[1]. Individuals prone to chronic inflammatory diseases have a greatly enhanced risk for cancer development and cancer patients with malignant tissues containing infiltrates of T_H2-polarized immune cells tend to have a worse clinical prognosis[2,3]. Epidemiological studies reported that inhibiting chronic inflammation in patients with pre-malignant disease or patients predisposed to cancer development has demonstrated chemopreventative potential[4-6]. These studies revealed that long-term usage of anti-inflammatory drugs, e.g. aspirin and selective cyclooxygenase (COX)-2 inhibitors significantly reduces cancer (breast, prostate, colon, renal, lung) risk[7].

Because of their enormous plasticity and capacity to produce a cytokines, chemokines, metallo- serine and cysteine proteases, reactive oxygen species (ROS), histamine and other bioactive mediators, chronically activated immune cells are key modulators of cell survival as well as regulators of ECM metabolism. Thus, physiologic processes necessary for tumor development, i.e., cell survival, tissue remodeling, and angiogenesis, are regulated by immune cells. This is exemplified by positive correlations between numbers of myeloid cells infiltrating human tumors with number of blood vessels[8,9], and experimental findings in mouse models where attenuating immune cell infiltration reduces angiogenesis and primary tumor development[10-14].

We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. To address these issues, we have taken several approaches to investigate mechanisms involved in: *i.* induction and maintenance of inflammatory microenvironments in premalignant and malignant tissues, *ii.* Assess the role of leukocyte and their proteases as regulators of cancer development, and *iii.* development of novel non-invasive imaging methodologies to monitor inflammation and/or deliver radiotherapeutics to carcinoma cells. Our studies are designed to test the hypothesis that *inflammation* is a critical parameter of neoplastic development and therefore represents an efficacious target for anti-cancer therapies.

II. RESEARCH ACCOMPLISHMENTS BODY:

Task 1. Define the profile and proteolytic contribution of leukocytes in human breast cancer and in transgenic mouse models of mammary carcinogenesis.

a. Elucidate the spectrum of $CD45^+$ cells in normal and neoplastic human breast tissues.

Months 1-12

A growing body of literature has emerged demonstrating that leukocytes functionally contribute to development of most human solid tumors. Leukocytic infiltrates in neoplastic stroma increase paralleling breast tumorigenesis, profiles of which vary but in general include both lymphoid and myeloid lineages[15,16]. We examined immunoreactivity of lineage-selective epitopes in archival paraffin-embedded tissue sections representing “normal” human mammary tissue as compared to ductal carcinoma in situ or frank carcinoma for: CD45 (leukocyte common antigen), CD68 (macrphages), chymase (mast cells), neutrophil elastase (neutrophils), CD4 (CD4+ T cells), CD8a (CD8+ T cells), CD20 (B lymphocytes) and CD31 (endothelial cells). This analysis revealed increased presence of B and T lymphocytes, macrophages, neutrophils and mast cells in premalignant and malignant tissue. These results were reported in our *2007 Annual Progress Report* and have now been partially published[15]

b. Elucidate the spectrum of $CD45^+$ cells in normal and neoplastic mammary tissues from mouse models of mammary carcinogenesis.

Months 1-24

The MMTV-PymT and MMTV-neu mouse mammary cancer models develop metastatic mammary tumors originating from ductal epithelial cells. These models share many of the hallmarks of multistage human breast cancer development including histological disease progression and immune cell infiltration[17-20]. In these models, as in human breast cancer, the number of $CD45^+$ positive leukocytes present in stroma surrounding neoplastic lesions increases paralleling disease progression. Though the total population of leukocytes parallels disease progression in both human and mouse tissue samples, the exact cell types present and their distributions have not been well defined. The MMTV-PymT model of mammary tumor development, developed by Muller and colleagues[20], represents a reasonable recapitulation of late-stage human breast cancer as determined by histological progression and expression of various biomarkers[18,21]. This model has also been

used to illustrate the contribution of macrophages to tumor development[13,22,23]. In our 2007 Annual Progress Report, we reported our initial characterization of immune cells in late stage carcinomas. In the previous 12 months, we have expanded these profiles and now provide more extensive analysis of premalignant and malignant lesions, pulmonary metastases, blood and spleen from animals bearing tumors at various stages as assessed by fluorescent activated cell sorting (FACS) (**FIGURE 1).** We assessed immune cell infiltrates in these distinct tissue locales utilizing flow cytometry and found distinct profile of innate and adaptive cells as compared to what we have previously found for tumors developing in either skin (data not shown) or lung (data not shown), indicating that organ-and microenvironmental specificity regulates chronic engagement of proinflammatory pathways. These data also reveal that a similar spectrum of immune cells is present in mammary tissue from MMTV-PymT as is found in human neoplastic mammary tissue.

c. Develop a profile of proteolytic activities in normal and neoplastic human breast tissues.
Months 1-12
This aim was completed in months 1-12 and reported in our 2007 Annual Progress Report

d. Develop a profile of proteolytic activities in normal and neoplastic mammary tissues from mouse models of mammary carcinogenesis.
Months 1-24
This aim was completed in months 1-12 and reported in our 2007 Annual Progress Report

e. Determine cellular origins of proteolytic activities in normal and neoplastic human breast tissues. (initial projection: Months 1-12; revised projection:
Months 24-36
As of month 24, comparative analyses between human and murine samples have not been initiated as we are still collecting human samples to increase our sample size of tissues reflecting DCIS and specific stages of disease. Since our experimental studies have revealed a functional role for the cysteine protease cathepsin C as a mediator of mammary carcinoma metastasis, we will focus on characterizing cathepsin C expression in human tissues once we have assembled a complete panel of tissues. Moreover, our studies of functional significance of CD4+ T cells have been quite successful (see below); thus, we have focused on these analyses at the expense of expanding our studies of leukocyte proteases for the time being.

f. Determine cellular origins of proteolytic activities in normal and neoplastic mammary tissues from mouse models of mammary carcinogenesis.
Months 1-24
Cathepsin C, also known as dipeptidylpeptidase I, is a lysosomal cysteine-class hydrolase expressed in most mammalian tissues. In myeloid cells and cytotoxic lymphocytes, it is found in secretory granules where it is solely responsible for NH_2-prodipeptide removal and catalytic activation of several leukocyte-derived serine proteases, including MC chymases and tryptases, neutrophil elastase (NE), cathepsin G, and cytotoxic lymphocyte-derived granzymes A and B[24-27]. Humans harboring loss of function mutations in the *cathepsin C* gene manifest several host immune defense deficits implicated in the pathogenesis of pre-pubertal aggressive peridontiti[28], Haim-Monk[29] and Papillon-Lefevre syndrome[30,31]. A broader role for cathepsin C has recently been revealed by examining immune responses in mice harboring homozygous deletions in the *cathepsin C* gene following pathologic challenge[24-27]. While cathepsin C-null mice exhibit normal neutrophil chemotactic responses to thioglycollate, they are resistant to experimental arthritis and neutrophil recruitment in response to zymosan and immune complexes, a defective response rescued by administration of a neutrophil chemoattractant[27] indicating that these myeloid cells are not compromised in their differentiation and can extravasate out of vasculature to infiltrate tissue. Together, these studies indicate that cathepsin C plays a significant role in regulating interstitial thrombin and fibronectin metabolism[32], angiogenesis[33-35], cytotoxic lymphocyte-mediated apoptosis, survival from sepsis[26] and experimental arthritis[27]. Based upon these novel findings, cathepsin C may represent a tractable target for therapeutic intervention in a diverse array of human disease states including cancer development.

Based upon the 7-fold increased mRNA expression of *cathepsin C* revealed by gene expression profiling analysis in tissues from MMTV-PymT mice **(reported in our 2007 Annual Progress Report**), we examined

tissue sections for cathepsin C protein expression and found that infiltrating immune cells are the predominant source of the enzyme (**FIGURE 2**). By utilizing double immunofluorescence staining, we have revealed that macrophages are the primary source of cathepsin C amongst the CD45+ cells infiltrating primary mammary carcinomas and metastatic lungs (**FIGURE 2)**.

g. Compare immune cell infiltrations in mouse and human normal and neoplastic tissues. Identify candidate cell types for further study.

Months 1-24

Based upon our histopathological examination of malignant human breast adenocarcinomas, as compared to our detailed analysis of adenocarcinomas from MMTV-PymT mice, we have compiled **TABLE 1.**

h. Compare proteolytic activities in mouse and human normal and neoplastic tissues based. Identify candidate protease activities for further study. (initial projection: Months 1-24; revised projection Month 24-48

As of month 24, these comparative analyses have not been initiated. We have recently developed an activity probe for cathepsin C[36]; however, obtaining unfixed, fresh human breast caner tissue has proven more difficult than initially thought. To resolve this issue, we have recently initiated a collaboration with Dr. Susan Love (Dr. Susan Love Medical Research Foundation) and Dr. Lisa Bailey (Alta Bates Hospital) to obtain freshly resected human breast cancer tissue via the UCSF Tissue Core and our existing CHR approvals. As we begin receiving these tissues, we will begin assessing cathepsin C activity and anticipate completion of this aim over the next 24 months.

Table 1. Comparison of leukocytic profiles in human breast cancer samples and mouse mammary adenocarcinomas from MMTV-PymT mice.

Leukocyte Population	Antigenic Marker Accessed H: human M: mouse	Normal Human Breast Tissue	Malignant Human Breast Tissue	Normal Murine Mammary Tissue	Malignant Murine Mammary Tissue
All leukocytes	CD45 (H/M)	+	++++	+	++++
Macrophages	CD68 (H/M)	+	+++*	+	+++
	F4/80 (M)	n.a.	n.a.	+	++++
	CD206 (H/M)	+/-	+/- to ++	++	+++
Granulocytes/ Myeloid cells	Neutrophil elastase (H)	+/-	++*	n.a.	n.a.
	GR-1 (M)	n.a.	n.a.	+/-	++
	7/4 (M)	n.a.	n.a.	+/-	++
Mast Cells	MCC activity (H/M)	+/-	+*	+/-	+/-
	cKit (H/M)	ND	ND	+/-	+
B cells	CD20 (H)	+/-	++*	n.a.	n.a.
	B220 (M)	n.a.	n.a.	-	-
CD4 T-helper	CD4 (H/M)	+/-	+++*	+/-	++
CD8 CTL	CD8 (H/M)	+/-	++*	+/-	+
T-regulatory	FOXP3 (H/M	+/-	+*	-	++

Table depicts average staining for various markers accessing varied leukocyte populations. Staining is graded – to ++++. – denotes no positive staining, +/- denotes sparse staining, + denotes few numbers of positive cells (<5% of total cells) and scale increases to ++++ which denotes extensive infiltration (>20% of cell number). n.a. denotes that staining is not possible due to cellular marker differences or lack of appropriate immunodetection reagent. ND denotes staining is possible but has not yet been completed. * denotes stainings with large degrees of variation between samples.

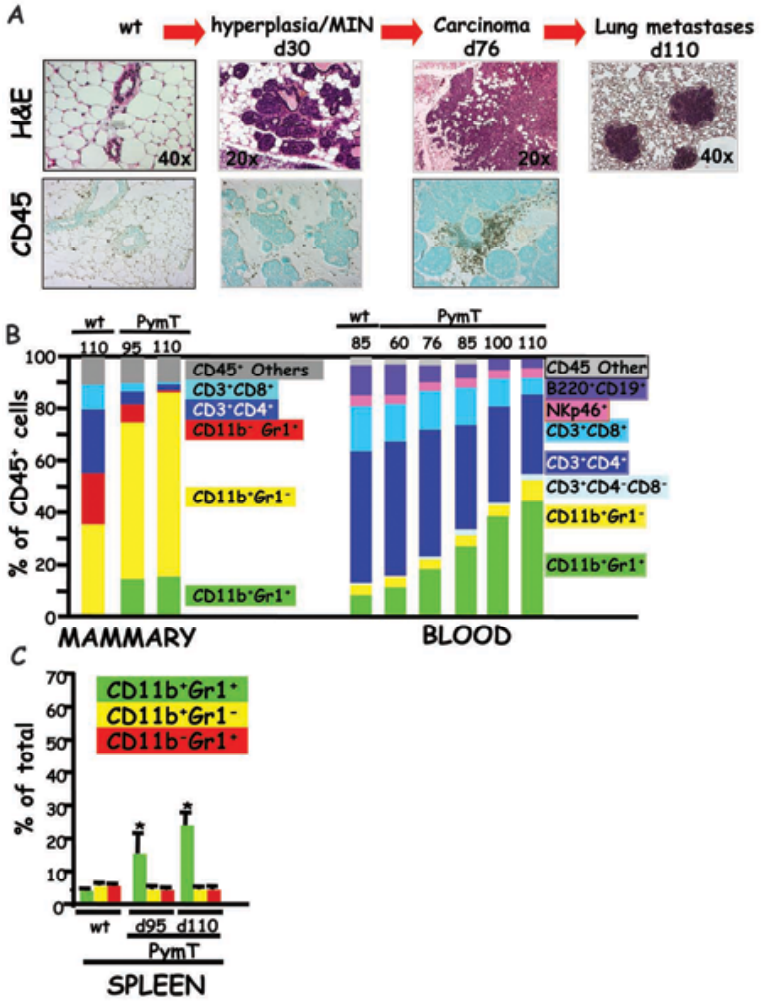


FIGURE 1: MMTV-PymT mouse model of mammary carcinogenesis. The MMTV-PymT model of mammary carcinogenesis has a step-wise progression to malignancy and pulmonary metastasis. **A)** Neoplstic stage can be visualized by hematoxylin & eosin staining and representative panels are shown of a normal gland (WT), pre-malignant/hyperplasia at day 30, malignant/invasive carcinoma at day 76 and pulmonary metastasis at day 110. Similar tissue sections stained by immunohistochemistry for CD45 (leukocyte common antigen, brown staining) are depicted in lower row. **B-C)** Accompanying neoplastic progression are changes in immune cell profiles in mammary tissue, blood **(B)** and spleen **(C).** FACS analysis of immune cell infiltrates from wild type (wt) mammary glands or PymT^{+} tumors (day 95 and 110) or blood drawn from wild type (wt) mice or tumor bearing mice (days 60-110) is depicted as the average % of immune cell types as a percentage of the total CD45^{+} population. C) FACS analysis of immune cells present in spleen tissue from wild type (WT) mice or tumor bearing mice (days 95 and 110) is depicted as the average % of immune cells of the total live cell population. Data are represented as means +/- SEM. In all sections * denotes statistical changes greater then $p<0.05$ by student t test.

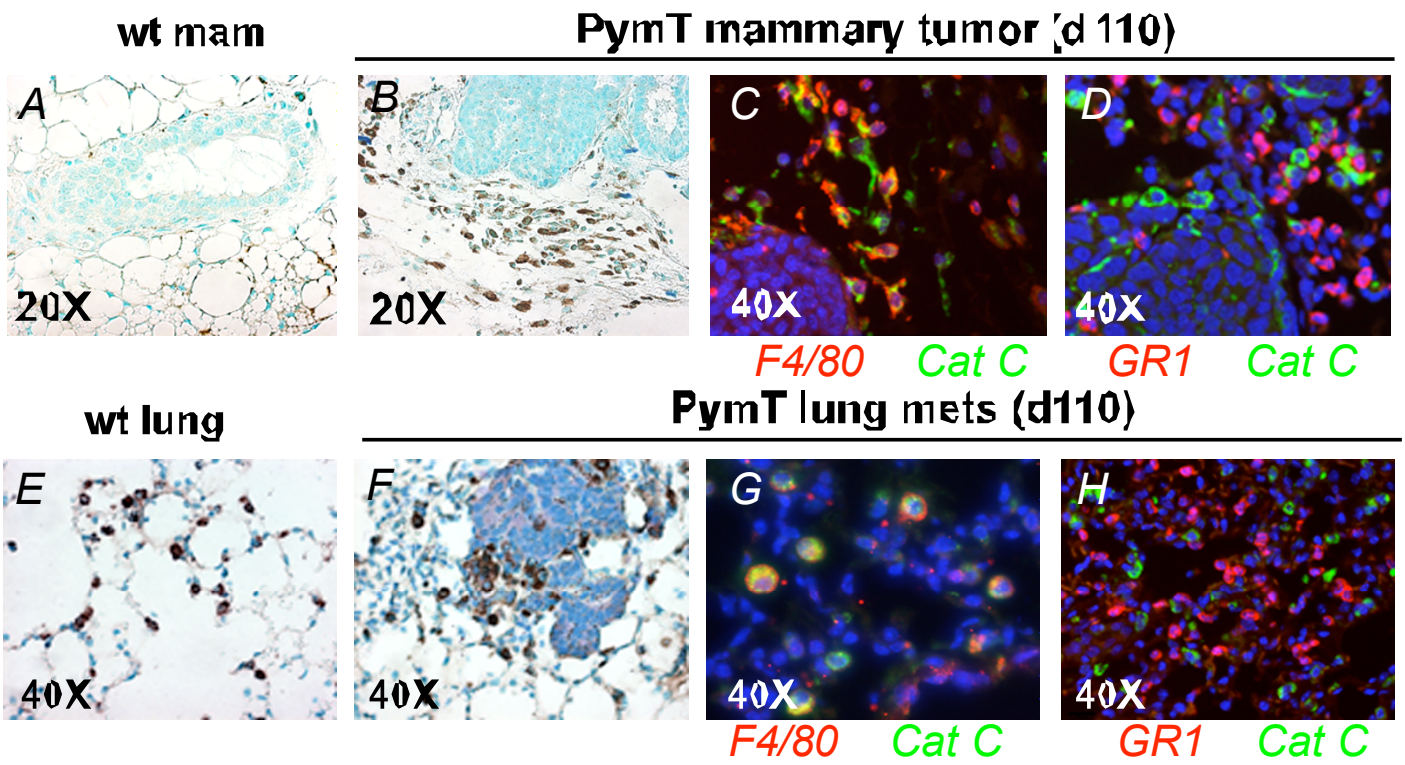


FIGURE 2: Immunodetection of cathepsin C in day 110 wildtype and MMTV-PymT mammary glands and lungs. Cathepsin C protein expression is detectable by immuno-detection in normal nontransgenic mammary (A) and lung (E) tissue (brown staining) and in MMTV-PymT mammary (B) and lung (F) tissue at d110. Dual immuno-fluorescent staining for cathepsin C (green) and either F4/80 C and G, red staining) or GR-1 (D and H, red staining) localizes cathepsin C expression to F4/80^{+} positive macrophages in malignant mammary tissue and metastatic lungs. Original magnifications are shown.

Task 2. Validate target molecules and/or specific immune cell types in biological assays and in animal models of mammary carcinogenesis.

a. Establish 3-dimensional mammary epithelial organotypic cell culture model system cultured on basement membrane.

Months 1-9

This aim was completed in months 1-9 and reported in our 2007 Annual Progress Report

b. Establish routine isolation and short-term culture conditions for mouse leukocytes.

Months 1-9

This aim was completed in months 1-9 and reported in our 2007 Annual Progress Report

c. Establish 3-dimensional organotypic co-culture model system for analysis of paracrine interactions between mammary epithelial cells and specific leukocytic cell types.

Months 6-12

This aim was completed in Months 6-12 as initially proposed and reported in our *2007 Annual Progress Report* and has now been expanded as shown below (**FIGURE 3**). We now routinely utilize this co-culture model system with primary cells to assess functionally significant paracrine interactions between diverse subsets of leukocytes and normal or PymT-initiated mammary epithelial cells (MECs)

d. Establish 3-dimensional organotypic culture model system for analysis of paracrine interactions between mammary epithelial cells and specific leukocytic-derived proteases.

Months 6-12

This aim was completed in months 6-12 and reported in our *2007 Annual Progress Report*

e. Analyze role of leukocytes in regulating mammary epithelial morphogenesis, proliferation, cell death, apicobasal polarity, cell-cell adhesion, and formation of invasive and protrusive structures in normal and oncogene-expressing 3-dimensional acini cultured on basement membrane.

Months 12-24

To reveal if CD4-dependent regulation of macrophage phenotype revealed from our *in vivo studies (see Task 2)* translated into an altered ability of primary mammary epithelial cells (MECs) to acquire invasive characteristics, a requirement for metastasis *in vivo*, we utilized an *ex vivo* three-dimensional (3D) organotypic co-culture model with primary MECs. MECs were isolated from either d76 PymT$^+$(pMEC) mice or 12-week old virgin negative littermates (nMECs) and placed in 3D overlay culture and allowed to form stable non-invasive structures that exhibit appropriate apical-basal polarity (data not shown) as previously described[37]. TAMs from PymT tumors were then isolated by FACS and added to 2-3-week old stable organoids (**FIGURE 3A**), in the presence or absence of T_H1 [interferon (INF)γ or lipopolysaccharide (LPS)] or T_H2-type [interleukin (IL)-4, IL-10 or IL-13] cytokines and organoid disruption in combination with formation of invasive structures quantitatively accessed (**FIGURE 3B**). Co-culture of TAMs and nMEC spheroids led to disruption and formation of invasive structures in a limited number of spheroids, activity that was significantly enhanced when TAMs were co-cultured with pMECs, where TAMs localized to invasive fronts of both nMEC and pMEC invasive structures (**FIGURE 3C**). Whereas neither nMECs nor pMECs exhibited any effect following addition of cytokines, IL-4 and IL-13 significantly enhanced ability of TAMs to induce invasive structures in pMEC spheroids (**FIGURE 3B)** whereas IL-10, INFγ or LPS significantly inhibited ability of TAMs to induce formation of invasive pMEC structures and instead conferred additional stability to organoid structures (**FIGURE 3B**). Similar results were found utilizing a standard boyden chamber invasion assays (data not shown). Together, these *ex vivo* data indicate that the ability of TAMs to induce invasive behavior in initiated MECs is dependent on the cytokines present in the local neoplastic microenvironment.

To evaluate how tumor-associated CD4$^+$ T cells factored into regulating TAM-induced organoid disruption, TAMs and pMECs were tri-cultured with CD4$^+$ T cells. In this tri-culture assay, TAMs again induced formation of invasive structures, activity that was significantly enhanced by presence of tumor-associated CD4$^+$ T cells in an IL-4-dependent manner (**FIGURE 3D**).

f. Analyze role of leukocyte-derived proteases in regulating mammary epithelial morphogenesis, proliferation, cell death, apicobasal polarity, cell-cell adhesion, and formation of invasive and protrusive structures in normal and oncogene-expressing 3-dimensional acini cultured on basement membrane.

Months 12-36

These assays have now been established as reported in our 2007 progress report; however, due to our focus on evaluating functional roles for leukocytes as regulators of mammary carcinogenesis, studies on protease function in vitro have been put on hold pending completion of our current leukocyte studies.

g. **Analyze role of candidate leukocytes by crossing mice deficient in or modified such that individual or classes of leukocytes (identified in Task 1) are deficient with transgenic mice prone to development of mammary adenocarcinoma.**

Months 1-48

As observed in several human neoplasms, human breast adenocarcinomas are characterized by infiltration of both innate and adaptive immune cells[15]. Given the critical role adaptive immune cells play in regulating innate immune cell responses during the initiation and progression of several inflammatory diseases (Gould et al. 2003, Lee et al 2002), and in some mouse models of carcinogenesis[12], we hypothesized that either B or T lymphocytes might play a role in regulating pro-tumor myeloid responses during mammary carcinogenesis. To assess this, we generated MMTV-Polyoma Middle T (PymT) mice harboring homozygous null mutations in key genes regulating differentiation and development of B and T cells, i.e., PymT/RAG1$^{-/-}$, and compared them for characteristics of neoplastic progression to PymT mice lacking B cells, i.e., PymT/JH$^{-/-}$, or selective subsets of T cells, i.e., PymT/CD4$^{-/-}$, PymT/CD8$^{-/-}$, or PymT/CD4$^{-/-}$/CD8$^{-/-}$ mice. Our analyses of these various cohorts revealed that neither complete nor selective lymphocyte-deficiency resulted in any significant difference in tumor latency, burden or histopathology of primary tumors (**FIGURE 4A-C**). In contrast, selective loss of CD4^{+} T lymphocytes in either RAG1$^{-/-}$, CD4$^{-/-}$/CD8$^{-/-}$ or CD4$^{-/-}$ mice, but not B or CD8^{+} T lymphocyte-deficient mice, resulted in significant attenuation in presence of pulmonary metastatic foci as well as reduced overall metastatic burden in lungs of PymT transgenic mice (**FIGURE 4D-E**), thus indicating that CD4^{+} T cells mediate mammary epithelial cell (MEC) metastasis to lungs.

To reveal if the decreased appearance of metastatic tumors in PymT/CD4$^{-/-}$ T cell mice reflected decreased ability of malignant MECs to exit primary tumors or perhaps instead reflected decreased ability of MECs to colonize lungs, we quantitatively assessed the frequency of circulating Pymt^{+} MECs in blood of tumor bearing PymT versus PymT/CD4$^{-/-}$ mice by RT-PCR analysis of PymT mRNA and flow cytometry evaluating CD45^{-}cytokeratin^{+} cells (**FIGURE 4F-G, respectively**). By both of these analyses, we found that in PymT/RAG1$^{-/-}$ and PymT/CD4$^{-/-}$ mice, there were significantly decreased numbers of circulating carcinoma cells when compared to heterozygous littermates indicating that the decreased metastatic capacity MECs from PymT/RAG1$^{-/-}$ and PymT/CD4$^{-/-}$ mice likely resulted from a CD4^{+} T lymphocyte-dependent dependent mechanism present within the primary tumor microenvironment.

CD4^{+} T cells regulate protumor properties of macrophages: Pollard and colleagues recently reported that macrophage deficiency in PymT mice significantly reduces appearance of pulmonary metastasis[13], essentially phenocopying our results with PymT/CD4 T cell-deficient mice (**FIGURE 4**). Based upon this similarity, we assessed immune cell infiltration of primary mammary tumors in 95 and 110 day-old cohorts of CD4-deficient/PtmT mice (**FIGURE 5**). These analyses revealed that recruitment of CD45^{+} immune cells into primary mammary tumors was unaffected by either CD4- or RAG1-deficiency (**FIGURE 5A**). Moreover, utilizing flow cytometry to quantitatively assess the spectrum of immune cell subtypes present in primary tumors, we found no significant change in either the composition of leukocytes infiltrating tumors (**FIGURE 5B**) or in presence of CD31^{+} endothelial cells (**FIGURE 5C**), thus indicating that CD4^{+} T lymphocyte-deficiency does not alter immune cell recruitment or development of angiogenic vasculature in primary mammary tumors.

Once in tissues however, mono-myelocytic cells express specialized and polarized functional properties as directed by the tissue and the immunological microenvironment – these correlate with classical T_H1 and T_H2 nomenclature and are often referred to as M1 or M2 polarized phenotypes, respectively. Classically activated M1 macrophages for example, are induced by T_H1 cytokines like interferon gamma (IFNγ or tumor necrosis factor alpha (TNFα) and granulocyte-monocyte-colony stimulating factor (GM-CSF), but are inhibited by

interleukin (IL)-4 and -13 that instead induce an M2 phenotype that is also accomplished by immune complexes, IL-1, -21, transforming growth factor beta (TGFβ), and glucocorticoid and secosteroid hormones. Since our assessment of immune cell composition of primary tumors revealed no significant change in mono-myelocytic cell recruitment based on presence or absence of $CD4^+$ T cells, we asked if instead, $CD4^+$ T cells were regulating polarization or activation status of recruited myeloid cells. To accomplish this, we evaluated cytokine production from $CD45^+/F4/80^+/Gr\text{-}1^-$ (tumor-associated macrophages, TAMs) and $CD45^+/Gr\text{-}1^+/CD11b^+/F4/80^{lo/-}$ (immature monocyte) isolated from mammary tumors removed from age-matched CD4-proficient versus CD4–deficient/$PymT^+$ mice. These two myeloid cell population were chosen because their recruitment has been previously reported to impact metastatic spread of $PymT^+$ tumors [13,14,38,39] and because they compose >80% of the inflammatory infiltrates found in late stage $PymT^+$ mammary adenocarcinomas. Analysis of M1 cytokine production in TAMs and immature monocytes from CD4-deficient tumors contained significantly elevated levels of several T_H1-type cytokines including IL-6 and IL-12, whereas TNFα and IL1β were found elevated in only the TAM population (**FIGURE 6A-B**). Analysis of IL-10 showed no difference in either population (**FIGURE 6E**). In contrast, expression other factors indicative of alternatively activated (M2) TAMs, including TGFβ and arginase were significantly reduced in TAMs isolated from CD4-deficient mice as compared to CD4-proficient age-matched littermate controls (**FIGURE 6F-G**). Taken together these data indicated that $CD4^+$ T lymphocytes regulate cytokine expression by TAMs

IL4 is an important T_H2-type cytokine produced by $CD4^+$ T cells and is known to differentially regulate myeloid cell effector functions. Thus, we assessed if the altered cytokine profile revealed in TAMs isolated from CD4-deficient/PymT mice was in part regulated by decreased IL4 in the tumor microenvironment. Using an *ex vivo* assay, we found that brief exposure of primary TAMs isolated from either PymT/$CD4^{-/-}$ or PymT/$RAG1^{-/-}$ mice to IL-4 resulted in a significant reduction in M1-type cytokines levels similar to that found in primary TAMs isolated from CD4-proficient/PymT mice (**FIGURE 6A-G**), thus indicating that T_H2-type cytokines like IL4 may repress M1 macrophage phenotype while simultaneously enhancing M2-type effector functions. Accordingly, we evaluated cytokine production by tumor-associated $CD4^+$ T cells from PymT mice and compared their expression characteristics for IL-4, INFγ and IL-17, as compared to $CD4^+$ T cells isolated from nontransgenic littermates following T cell receptor (TCR) activation, and found that indeed tumor-associated $CD4^+$ T cells produce less INFγ and IL-17 than naïve $CD4^+$ T cells (**FIGURE 7**). Moreover, by flow cytometry, we found that a higher percentage of tumor-associated $CD4^+$ T cells produce IL-4 than those that produce INFγ or IL-17 (data not shown). This difference appears to be significant with regards to regulating TAM cytokine expression since when $CD4^+$ T cells (isolated from PymT tumors) are co-cultured with either LPS-stimulated TAMs, or TAMs isolated from PymT/$CD4^{-/-}$ mice, there is a dose-dependent decrease in T_H1-type cytokine production. Taken together, these data support the notion that tumor-associated $CD4^+$ T cells are T_H2 polarized and produce IL-4 following activation, and that the increased presence of IL-4 in the tumor microenvironment regulates pro-tumor properties of TAMs.

h. **Analyze role of proteases expressed by both tumor and stromal cells by crossing transgenic mice deficient in a protease already implicated in breast cancer progression with transgenic mice prone to development of mammary adenocarcinoma.**
i. **Analyze role of proteases expressed by leukocytes by crossing transgenic mice deficient in a candidate leukocyte identified in Task 1 with transgenic mice prone to development of mammary adenocarcinoma.**

Months 1-24 (h-i)

To assess the functional significance of cathepsin C as a regulator of mammary carcinogenesis, we have generated breeding colonies of PymT mice harboring homozygous null mutations in the cathepsin C gene and compared primary tumor development and pulmonary metastasis in age matched cohorts of PymT/cathepsin $C^{+/-}$ and PymT/cathepsin $C^{-/-}$ mice. Our data reveal that primary tumor latency and total primary tumor burden (**Figure 8A)** in $PymT^+$/cathepsin $C^{+/-}$ and PymT/cathepsin $C^{-/-}$ mice (>20 mice/experimental group) was unchanged where tumor size was determined by caliper measurement and multiple tumors in one animal summed together (data not shown) However, we have found that cathepsin C does significantly regulate pulmonary metastasis formation as shown in **FIGURE 8B-D.** We found that upon quantitatively assessing the average number of metastatic foci from 5.0 μm lung sections/mouse from 110 day old $PymT^+$/cathepsin $C^{+/-}$

and PymT/cathespin $C^{-/-}$ mice where each lung was completely sectioned and 6 sections evaluated every 100 μm apart by staining with H&E and total number of metastatic foci (greater then 5 cells) was quantified a statistically significant difference was found ($p<0.05$) by student t test (**FIGURE 8B-C)**. In addition, we revealed that the change in metastatic frequency was likely due to a change within the primary tumor microenvironment.

We assessed the number of circulating carcinoma cells by FACS following blood draw by right heart puncture of terminal day 110 animals and evaluated live, cytokeratin positive, CD45 negative cells and found a statistical changes greater then $p<0.05$ as determined by student t test **(FIGURE 8D)**.

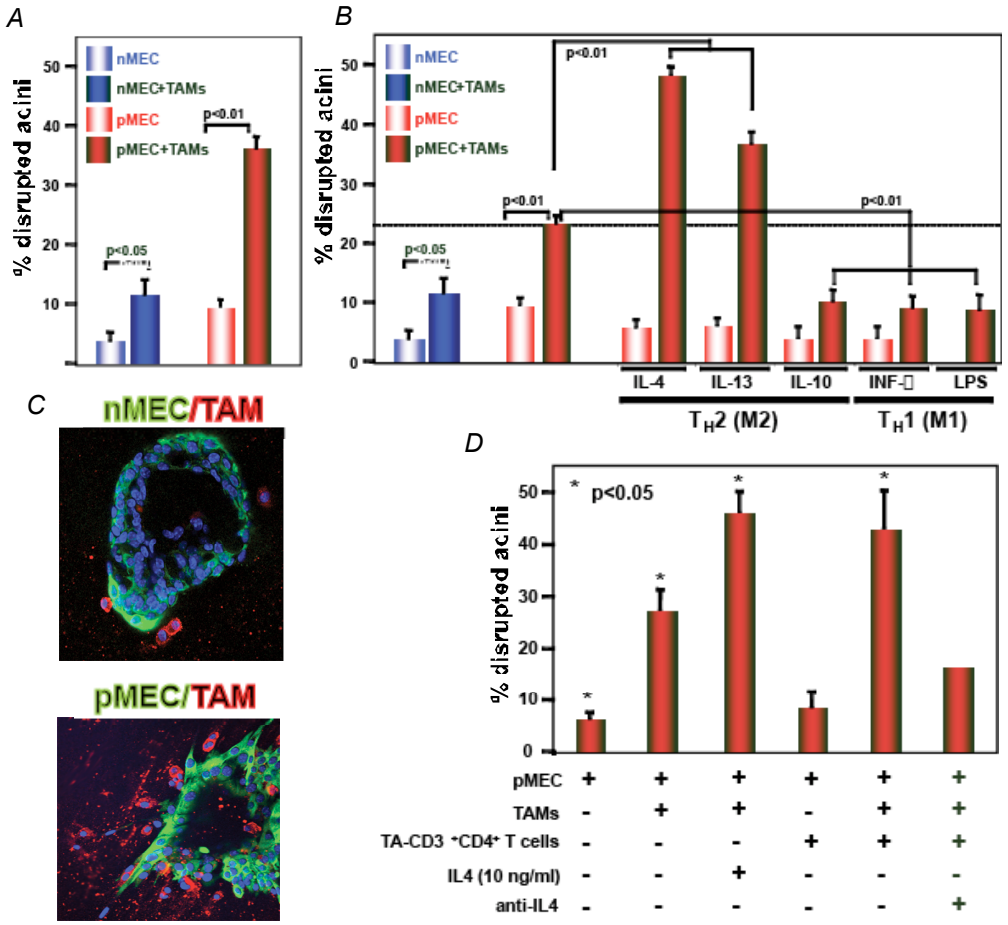
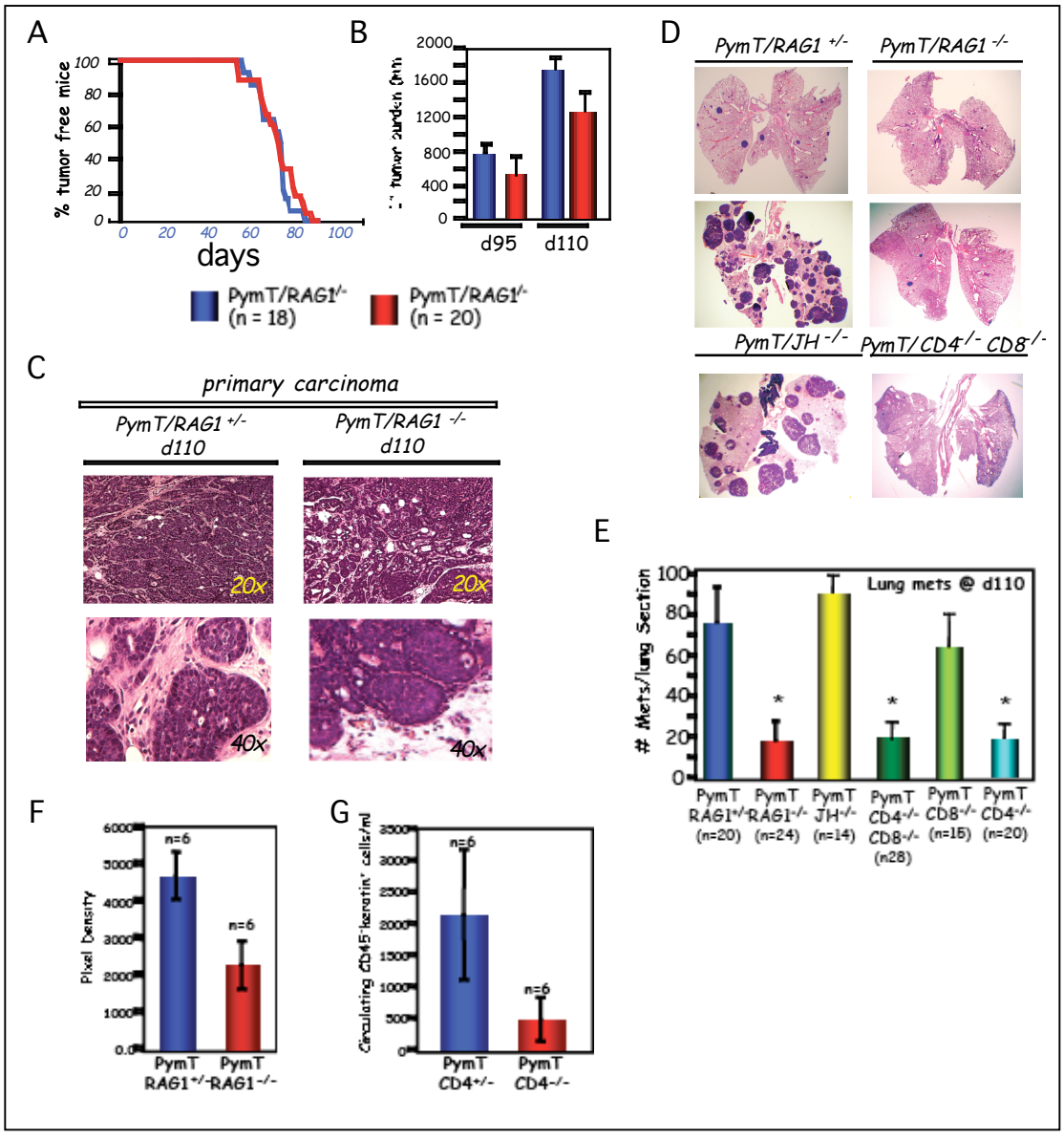


FIGURE 3: 3D co-culture of primary murine mammary epithelial cells and leukocytes.

A) Quantitation of acinar disruption following co-culture of TAM (48 hours) with nMEC or pMEC (mammary epithelial cells from MMTV-PymT mice); disrupted acini are counted and then expressed as a percentage of total acini present. Data are represented as means +/- SEM. **B)** Quantitation of acinar disruption following co-culture of TAM (48 hours) with pMECs, in the presence of either vehicle, IL-4 (20n g/ml), IL-13 (20 ng/ml), IL-10 (20 ng/ml), INFγ (20 ng/ml) or LPS (50 ng/ml). Disrupted acini are counted and then expressed as a percentage of total acini present. Data are represented as means +/- SEM. **C)** Representative images of pMECs acini and TAMs in co-culture accessed by Immuno-fluorescent staining for detection of Keratin 7 (Green: epithelial cells), F480 (Red: macrophages) and DAPI (Blue: DNA). **D)** Quantitation of acinar disruption of 14 days old pMEC acini following co-culture (48 hours) with TAMs and/or tumor-derived $CD3^+/CD4^+$ T-cells. Co-cultures are also treated with recombinant mouse IL-4 (10 ng/ml) and/or an anti-mouse IL-4 neutralizing antibody (0.5 mg/ml; clone OP06) added 12 hours prior to leukocytes. Disrupted acini are quantitated and graphed as a percentage of total acini. Data are represented as means +/- SEM. In all sections * denotes statistical changes greater then $p<0.05$ by student t test, if not otherwise specified.

FIGURE 4: CD4+ T cells regulate pulmonary metastasis of mammary adenocarcinomas

A) Kaplan Meyer analysis tumor incidence of PymT^{+}/RAG1$^{+/-}$ (blue) versus PymT^{+}/RAG1$^{-/-}$ (red) mice (20 mice/group) is depicted as % of tumor free animals. Animals were considered to be tumor-free until a palpable mass (>4.0 mm) persisted for longer then 4 days. **B)** Total tumor burden of PymT^{+}/RAG1$^{+/-}$ (blue) versus PymT^{+}/RAG1$^{-/-}$ (red) mice shown as mm^3, with 20 mice/groups. Tumor size was determined by caliper measurement and all tumors per animal were summed. Data are represented as means +/- SEM. **C)** Representative mammary adenocarcinoma tissue sections from 110 day old PymT^{+}/RAG1$^{+/-}$ versus PymT^{+}/RAG1$^{-/-}$ mice stained by H&E taken at 20x and 40x magnification. **D)** Representative lung tissue sections depicting metastatic tumor burden from 110 day old PymT^{+}/RAG1$^{-/-}$, PymT^{+}/JH$^{-/-}$, PymT^{+}/CD4$^{-/-}$/CD8$^{-/-}$ stained by H&E taken at 5x magnification. **E)** Quantitation of average number of metastatic foci/5.0 μm lung section/mouse from 110 day old PymT^{+}/RAG1$^{-/-}$, PymT^{+}/JH$^{-/-}$, PymT^{+}/CD4$^{-/-}$/CD8$^{-/-}$ mice. Each lung was completely sectioned. 6 section 100 μm apart were stained by H&E and the total number of metastatic foci (greater then 5 cells) was quantified. Each of the six sections were averaged/mouse and each bar represents >20 mice. Data are represented as means +/- SEM and * denotes statistical changes greater then p<0.05 by t test. **F)** Quantitation of low cycle RT-PCR for PymT mRNA transcript. PCR was run for 28 cycles and band intensity was quantitated using BioRad Image Ready software. Data are represented as means +/- SEM. **G)** The number of circulating carcinoma cells access by FACS on blood draw from right heart puncture. 200 μl of blood was accessed for cytokeratin positive, CD45 negative live cells. Data are represented as means +/- SEM. In all sections * denotes statistical changes greater then p<0.05 by student t test.



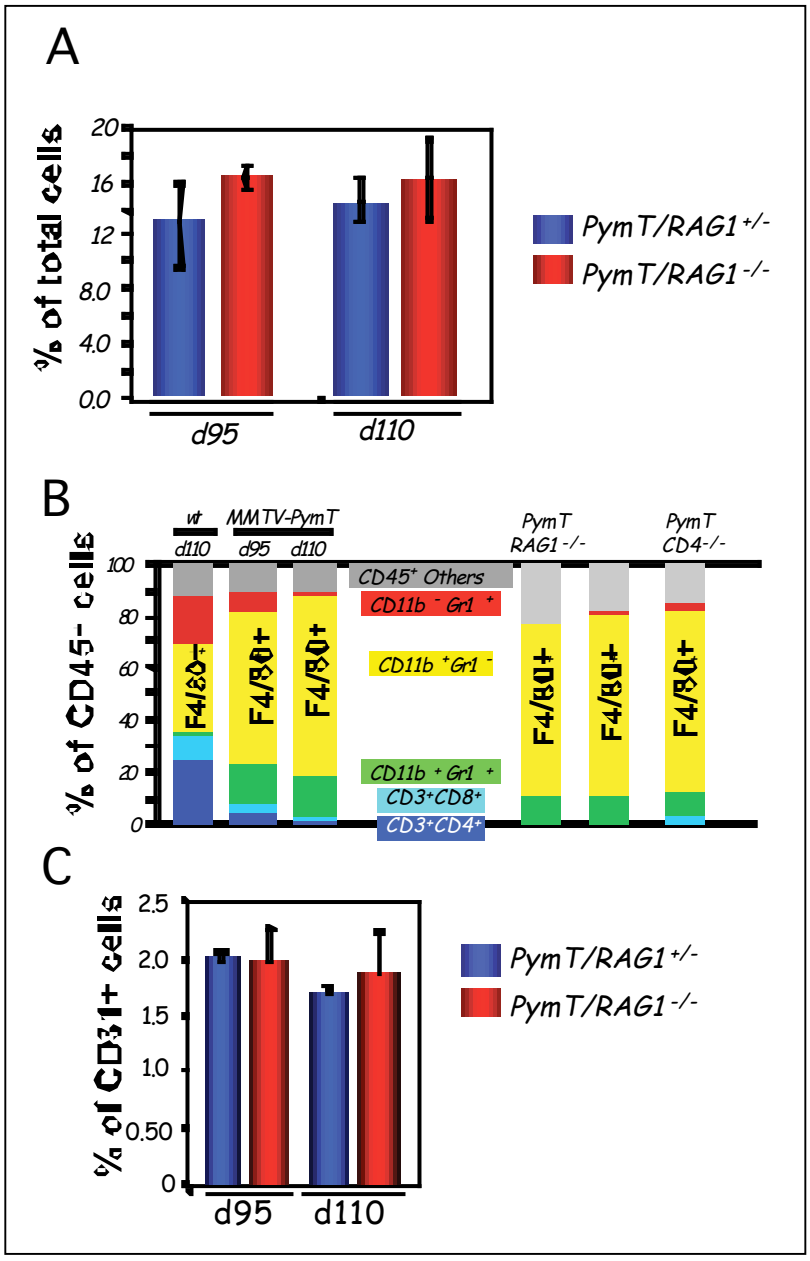


FIGURE 5: Immune cell infiltration and endothelial expansion in the absence of adaptive immunity. Changes in immune cell infiltrate and endothelial expansion were accessed by flow cytometry in MMTV/PymT mammary adenocrcinomas. **A)** Analysis of $CD45^+$ leukocytes into primary tumors from 95 and 110 day old PymT/$RAG1^{+/-}$ and PymT/$RAG1^{-/-}$ mice depicted as average % of immune cells of the total live cell population. **B)** FACS analysis of immune cell infiltrates from wt mammary glands or tumors from PymT/$RAG1^{+/-}$, PymT/$RAG1^{-/-}$, PymT/$CD8^{-/-}$, and PymT/$CD4^{-/-}$ mice (day 110) depicted as the average % of immune cells as a percentage of the total $CD45^+$ population. **C)** FACS analysis of CD31+ endothelial cells in tumors from 95 and 110 day old PymT/$RAG1^{+/-}$ and PymT/RAG1 mice are depicted as the average % of cells of total live cell population. Data are represented as means +/- SEM.

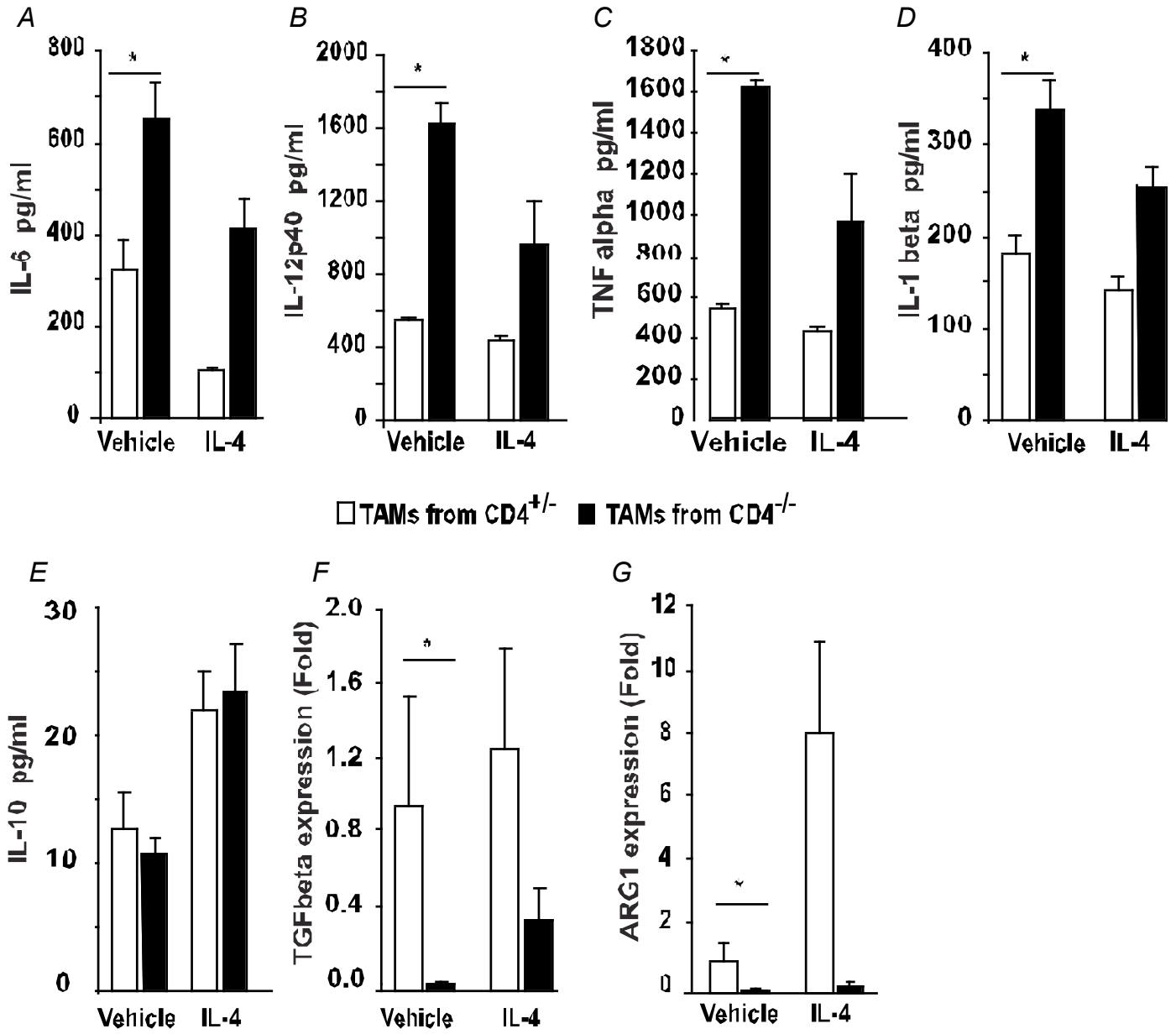


FIGURE 6: Cytokine profiles of TAMs from Pymt/CD4-deficient mice are shifted towards an M1 phenotype. A-E) Cytokine expression analysis from TAM conditioned media. Tumor associated macrophages were isolated by flow sorting from mammary tumors from 95 day old PymT/CD4$^{+/-}$ and PymT/CD4$^{-/-}$ mice. Cytokine expression was accessed by ELISA analysis of conditioned media from TAMs after 18 hours culture, with or without recombinant IL-4 (20 ng/ml). Data are represented as means +/- SEM and * denotes statistical changes greater then $p<0.05$ by student t test. **F-G)** Quantitative real-time PCR analysis of TGFβ and arginase expression in tumor associated macrophages isolated by flow sorting from mammary tumors from 95 day old PymT/CD4$^{+/-}$ and PymT/CD4$^{-/-}$ mice. Fold changes were accessed using a delta ct method and normalizing to β-actin. Data are represented as the mean fold change from the standardized sample (CD4+/-Veh) +/- SEM and * denotes statistical changes greater then $p<0.05$ by student t test.

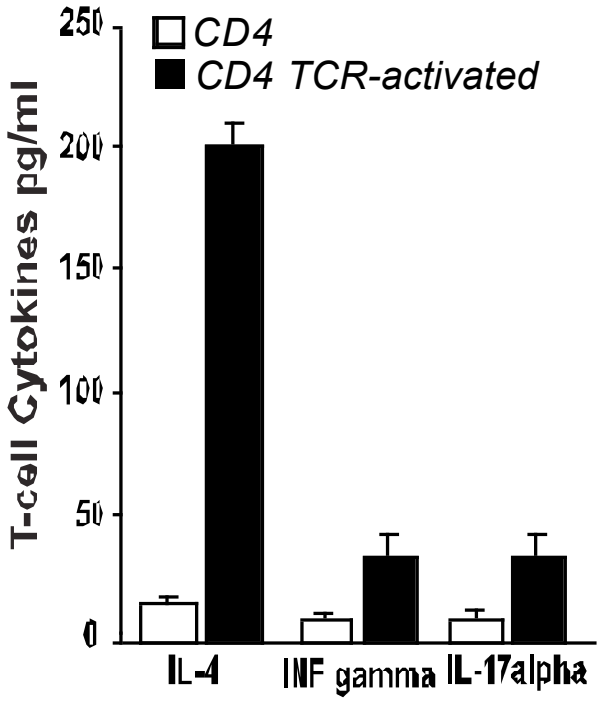


FIGURE 7: Cytokine profiles of tumor-associated CD4^{+} T cells from PymT mammary carcinomas. Tumor associated CD4 lymphocytes were isolated by flow sorting from mammary tumors from 95 day old PymT/CD4$^{+/-}$ mice. Cytokine expression was accessed by ELISA analysis of conditioned media from CD4 T cells after 18 hours culture, with (blck bars) or without (white bars) TCR activating antibodies anti-CD3 (200 ng/ml) and anti-CD28 (100 ng/ml). Data are represented as means +/- SEM and * denotes statistical changes greater then $p<0.05$ by student t test.

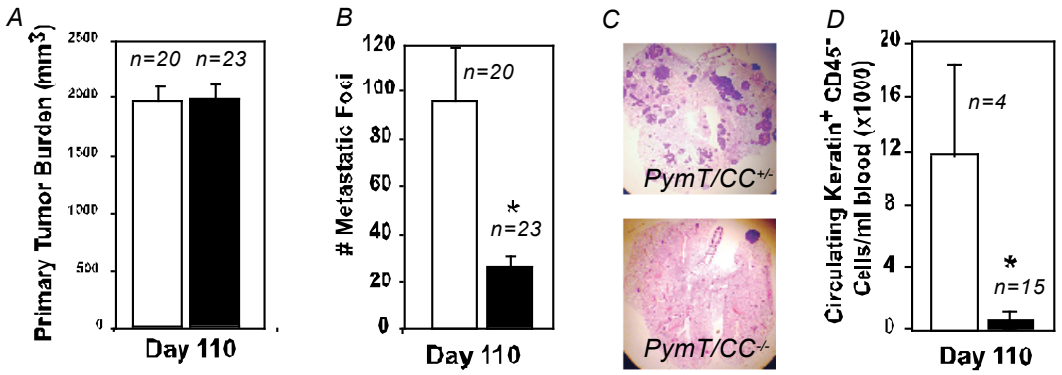


FIGURE 8. Cathepsin C promotes metastatic spread but not primary tumor development. A) Total primary tumor burden in PymT^{+}/cathepsin C$^{+/-}$ and /cathepsin C$^{-/-}$ mice shown as mm^3, >20 mice/experimental group at day 110. Tumor size was determined by caliper measurement and multiple tumors in one animal summed together. Data are represented as means +/- SEM. B) Quantitation of average number of metastatic foci/5.0 μm lung section/mouse from 110 day old PymT^{+}/cathepsin C$^{+/-}$ and PymT/cathespin C$^{-/-}$ mice. Each lung was completely sectioned and 6 sections 100 μm apart were stained by H&E and the total number of metastatic foci (greater then 5 cells) was quantified. Each of the six sections was averaged per mouse and each bar represent >20 mice. Data are represented as means +/- SEM and * denotes statistical changes greater then $p<0.05$ by student t test. C) Representative lung sections depicting metastatic tumor burden from 110 day old PymT^{+}/cathepsin C$^{+/-}$ and PymT/cathepsin C$^{-/-}$ mice stained by H&E taken at 5x magnification. D) The number of circulating carcinoma cells access by FACS on blood draw from right heart puncture. 200 μl of blood was accessed for live, cytokeratin positive, CD45 negative cells. Data is represented as means +/- SEM and * denotes statistical changes greater then $p<0.05$ by student t test.

Task 3. Develop non-invasive imaging reagents to monitor leukocyte and/or protease-specific events during mammary carcinogenesis

a. **Identify and characterize selective peptide substrates and selective binding peptides for proteases already implicated in breast cancer progression. (initial projection: Months 1-24; revised projection:**

Month 1-36

Targeted cancer radiotherapy requires highly selective radiolabeled carriers that provide efficient radionuclide delivery. Radiotherapeutics incorporating sophisticated selective cell-targeting strategies are promising, but few if any current radiotherapeutics have an optimal balance of long intra-tumoral residence times and adequate clearance from nontarget sites, and many suffer *in vivo* catabolism or metabolism of the radionuclide delivery molecule.

In order to selectively target a tumor, deliver a cytotoxic dose of radioactivity to tumor cells, and minimize non-specific radiation to non-tumor tissues, in collaboration with our colleague Dr. Benjamin Franc in the Department of Radiology (UCSF), we helped to develop a radiotherapeutic using a novel targeting strategy. The radionuclide delivery molecule (RDM) developed in this project targeted matrix metalloproteinase-14 (MMP14), a protease that is up-regulated in many breast cancers (**see 2007 Annual Report**). Each probe contains a poly-D-arginine cell-penetrating peptide, attached to an MMP14 cleavable peptide sequence, and a negatively-charged attenuation sequence. In our rational design approach, we employed an *in silico* screen to map the equilibrium conformations of four tested probe candidates, using an atomistic AMBER7 force field combined with GB/SA implicit solvation and modern conformational sampling techniques. In addition, we performed parallel synthesis of four probes and incorporated a bis-pyridyl chelate for labeling of ^{99m}Tc. Cleavage of probes by MMP14 and the generation of fragments capable of translocation across a cell membrane were demonstrated in cultured breast carcinoma cells (**FIGURE 9**). We performed initial in vivo optical imaging studies of the RDM using MDA-MB-231 cells transfected to overexpress MMP-14 as described above, where cells were harvested using trypsin/EDTA, and suspended in calcium- and magnesium-free Hank's balanced salt solution. Greater than 90% cell viability was established prior to implantation of 10^5 cells into the right flank of immunodeficient RAG1$^{-/-}$ mice (N=3). The procedure was repeated to implant 10^5 vector control (non-MMP-14-expressing) cells into the left flank of the same RAG1$^{-/-}$ mouse as a control. When the tumors reached approximately 1.0 cm in diameter, mice were anesthetized with isoflurane gas and were repeatedly microinjected across the tumor with a 1.0 µM solution of the BODIPY RDM prototype described above. After four hours, the mice were sacrificed, and the tumors were harvested and sectioned. Following DAPI staining, the tumors were imaged by fluorescence microscopy (Leica DC500, 546 nm emission filter.) Washout of the fluorescent BODIPY RDM from the tumor was seen in all vector control xenografts while retention of the agent was seen in the MMP-14 overexpressing xenografts at 4 hours (**FIGURE 10**). The levels of fluorescence were significantly different between the MMP-14 expressing xenografts and the vector controls, demonstrating that retention of the agent in the tumor tissue is dependent upon the expression of MMP-14.

Molecular modeling was found to predict the efficiency of cell-penetrating peptide attenuation and protease cleavage of the peptide substrate. Utilizing kinetic data on MMP-14 cleavable substrates available in the literature and previous work in the Franc lab on appropriate PTD attenuators, we selected two peptide sequences on which to focus our RDM synthetic efforts: DGDGDGD-GG-SGRIGF^LRTA-GG-r8-SAAC (highly sensitive to cleavage by MMP-14 but also cleaved to some extent by MMP-2 and MMP-9) and DGDGDGD-GG-SGRSEN^IRTA-GG-r8-SAAC (selectively cleaved by MMP-14) where D, G, S, R, I, F, L, T, and A are all standard abbreviations for L-isomers of the amino acids, r8 is an octamer of D-arginine, and SAAC is a single amino acid chelate. "DGDGDGD" is considered the PTD-attenuation domain, "r8" is the PTD, "SGRSENIRTA" is the MMP-14 cleavable linker of these two domains, and "^" represents the point of expected cleaving by MMP-14. A schematic of this construct was provided in the 2007 Annual Report. We had anticipated completing a manuscript by month 24 based on our collaboration with Drs Franc and Watkins; however, unfortunately, in month 17, both Drs. Franc and Watkins abruptly departed UCSF leaving our manuscript unfinished. While we will attempt to complete the final experimental details for this manuscript, we are uncertain as to how long this process will require as synthetic chemistry is not our expertise. As such, we are exploring other options for finishing this aspect of the project with other investigators at UCSF and hope to provide more final details by out year 3 Progress Report.

b. **Identify and characterize selective peptide substrates and selective binding peptides for candidate proteases validated in Task 2.**

Months 24-48

Based upon our compelling data implicating cathepsin C as an important regulator of late-stage mammary carcinogenesis, we generated with our collaborator Matt Bogyo (Stanford University)[36] a novel highly selective activity-based probe to assess relative changes in cathepsin C activity in tissue lysates isolated from distinct stages of neoplastic progression. We are currently utilizing this probe to assess cathepsin C activity in tissue lysates from distinct stages of progression in MMTV-PymT mice, and we are in the process of re-synthesizing a new fluorescent probe, as was done previously by the Bogyo group for evaluating cathepsin B in pancreatic islet tissue[40], to evaluate relative cathepsin C activity in tissue ex vivo. For the reasons mentioned above regarding our focus oer the previous 12 months on investigating leukocytes during mammary carcinogenesis, this aspect of the project was not pursued vigorously over the previous 12 month period. We anticipate its completion by month 48 as initially projected.

c. **Synthesize novel fluorescent probes for imaging protease activity using peptide substrates identified above (3.a.).**

Months 6-36

These studies will be pursued following identification of a new collaborator to replace Drs Franc and Watkins.

d. **Demonstrate ability of fluorescently-labeled molecules to localize to xenograft and/or 3-dimensional organotypic cancer models using confocal fluorescence and/or whole-body fluorescence imaging.**

Months 6-36

To initiate these studies, and based on out compelling preliminary data demonstrating functional role for monocytes in regulating late-stage mammary cancer metastasis, we have investigated whether fluorescently-labeled monocytes could be used for optical imaging of peri-tumoral inflammation since non-invasive evaluation may have diagnostic value and assist with therapeutic monitoring. Thus, 5- to 10 million murine monocytes were labeled with DiD (a fluorescent carbocyanine dye) and injected intravenously into MMTV-PymT tumor-bearing mice and age-matched negative littermate control mice. Optical imaging was performed before, directly after, and at 1, 2, 6 and 24 hours after cell injection. Uptake in body organs was evaluated to determine technical success. Ratios of post-injection to pre-injection fluorescent signal intensity (SI post/pre) of the tumors (MMTV-PymT mice) and mammary tissue (/n controls) were calculated. Confocal fluorescent microscopy was used to confirm that labeled cells were present within tumors after intravenous injection.

Technical success was obtained in all 12 mice, with increasing fluorescence seen in liver, spleen and lungs of all animals after labeled-monocyte injection. MMTV-PymT mice showed progressing tumor fluorescence up to 24 hours after cell injection, with average SI post/pre ratios of 1.8+/- 0.2 (range, 1.1-2.6). Control mice showed no increased fluorescence in mammary tissue after monocyte injection with average SI post/pre ratios of 1 +/- 0.0 (range, 1.0 to 1.0) (**FIGURE 11**). The difference between averages was found to be statistically significant, with a p-value of 0.01. Confocal fluorescence microscopy confirmed the presence of intravenously injected DiD-labeled cells within the breast tumors (**FIGURE 12**). From these initial studies, we conclude that i.v. delivery of congenic monocytes will accumulate at site of mammary cancer development in PymT mice, providing proof of principle that peri-tumoral inflammation can be evaluated using optical imaging. Further studies will be needed to clarify if this novel imaging parameter will provide prognostic or therapeutic information and to what degree we can target specific subpopulations of leukocytes in primary tumors as opposed to pulmonary metastases.

e. **Identify and characterize covalently coated iron oxide nanoparticles with poly(ethylene glycol) polymers functionalized with specific peptides or antibodies that uniquely interact with target immune cell populations. Months 12-48.**

f. **Validate covalently coated iron oxide nanoparticles with poly(ethylene glycol) polymers functionalized with specific peptides or antibodies for selectivity in the organotypic 3-dimensional co-culture models. Months 12-48.**

g. **Validated covalently coated iron oxide nanoparticles with poly(ethylene glycol) polymers functionalized with specific peptides or antibodies *in vivo* in the parental, immune and/or protease-modified mouse models of mammary carcinogenesis. Confirm relationship between level of nanoparticle uptake within tissues and level of immune cell infiltration histologically. Months 12-48.**

h. **Analyze evolution of leukocyte infiltration and/or protease expression in mouse mammary models using fluorescently-labeled molecular probes where animals are imaged longitudinally on weekly intervals. Months 12-60.**

i. **Select candidate molecular probes emerging from above (3.d.) that demonstrate the capability of being detected *in vivo* and are present at key timepoints in the evolution of breast cancer and use as a platform for development of protease-specific radiolabeled probes for single photon emission computed tomography (SPECT). Months 12-60.**

j. **Demonstrate ability of agents in 3.i. to localize to tumors in proportion to the level of the specific protease targeted using *in-vivo* SPECT-CT imaging (X-SPECT, Xenogen Corp.) with *ex-vivo* autoradiography, scintillation well-counting, and immunohistochemistry to pathologically confirm levels of tissue expression and/or immune cell infiltration in areas concentrating the radiolabeled probe. Months 24-60.**

k. **Monitor relationships between presence of various immune cell populations and protease expression within mouse mammary models utilizing multi-modality imaging (MRI, SPECT-CT) in combination with the immune cell (MR) agents and protease (SPECT-CT) agents developed in 3.e. and 3.i., respectively. Months 36-60.**

These studies (e-k) have not yet been initiated

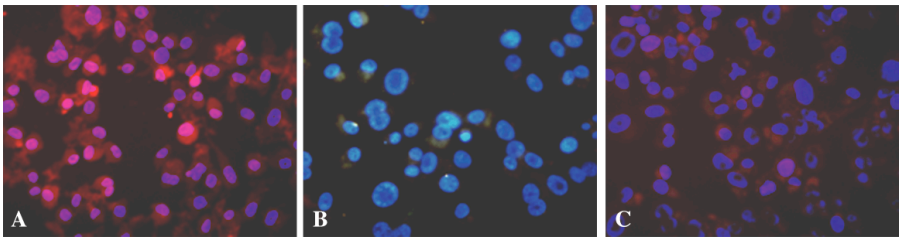


FIGURE 9. Imaging MMP-14 expressing cells. MDA-MB231 cells stabley expressing MMP14 (A) or vector control (B) were incubated in serum-free medium with 1.0 μM of BODIPY-labelled MMP14-probe for 4-hr MDA-MB231-MMP14 cells were incubated with 1.0 μM free BODIPY dye as negative control (C). Cytoplasms of MDA-MB231-MMP14 cells show increased fluorescence due to internalization of penetration peptide following MMP-14 cleavage at

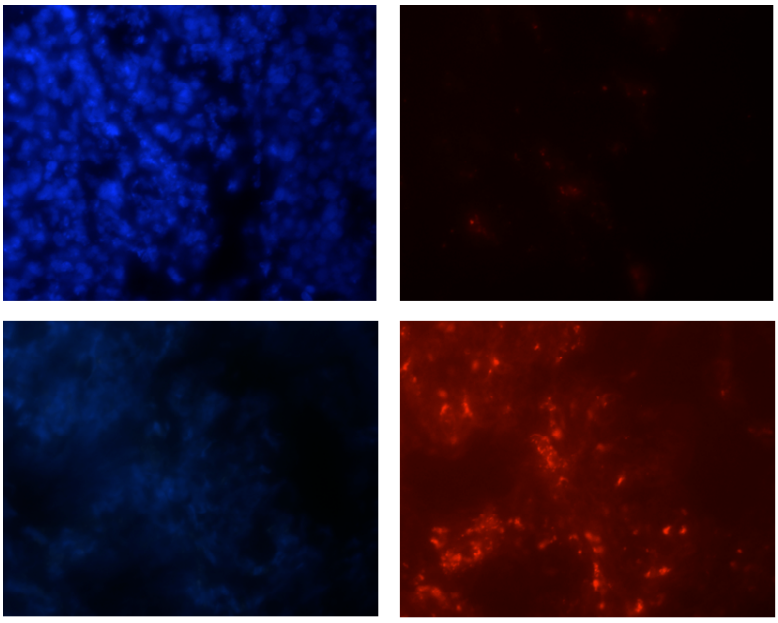


FIGURE 10: Imaging MMP14 expressing tumor xenographs. Sections of vector control MBA-231 xenograft demonstrating nuclear staining by DAPI (A) versus the minimal fluorescence at the BODIPY emission (low RDM retention (B), as compared to tissue sections from MMP-14 expressing MBA-231 xenograft tumors showing nuclear staining by DAPI (C) various intense fluorescence emission at the BODIPY emission (high RDM retention) (D)

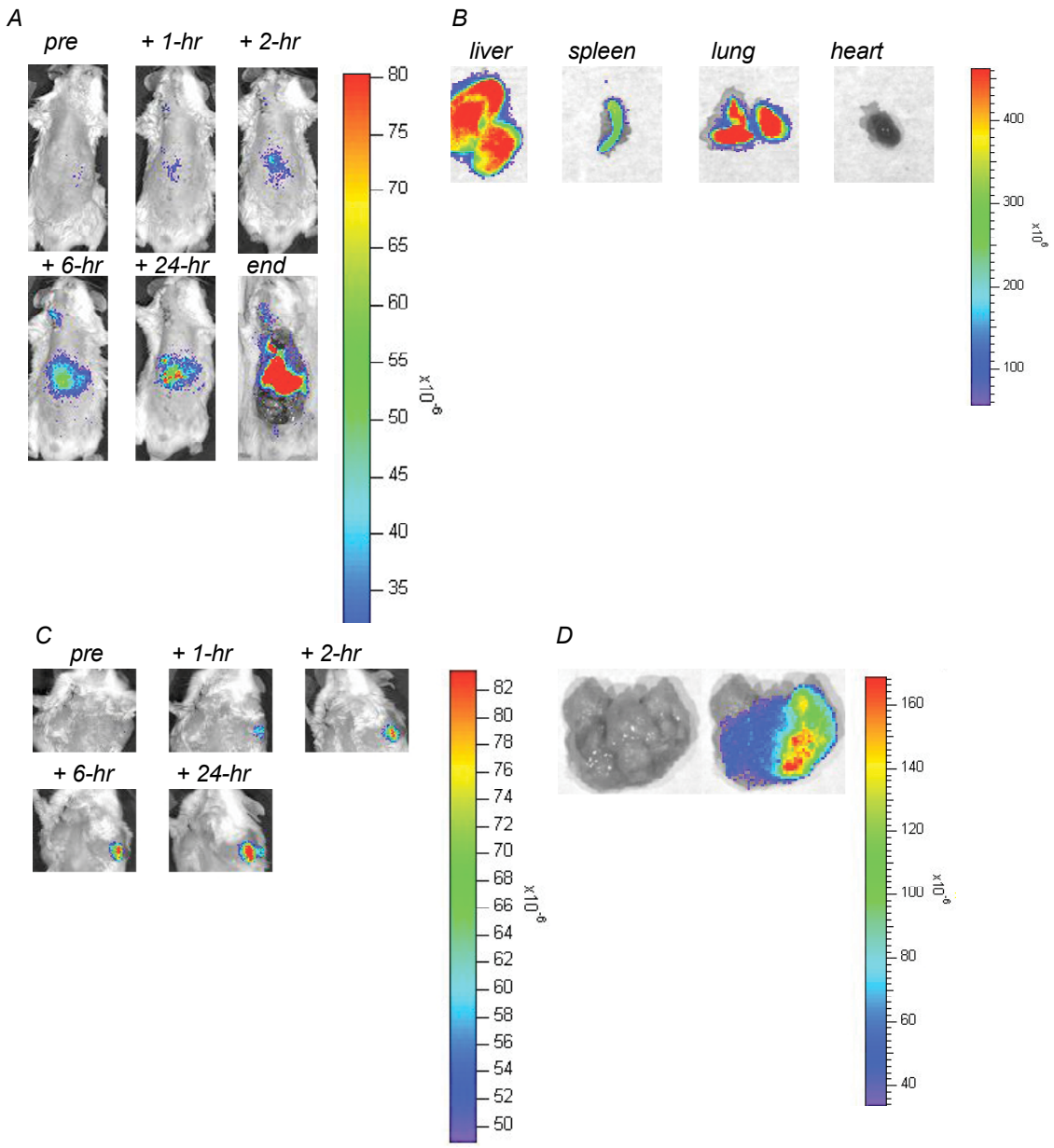


FIGURE 11: *In vivo* optical imaging of labeled monocytes. A. *In vivo* optical imaging of a control mouse after i.v. injection of DiD-labeled monocytes. Top row, left to right: pre-injection, 1-hr, and 2-hr post-injection. Bottom row, left to right: 6-hr, 24-hr post injection, and post-mortem dissection. **B.** Removed organs 24-hr post injection. Left to right: Liver, spleen, lungs, heart. Images are representative of control mice (n=6) injected with DiD-labeled monocytes. **C.** *In vivo* optical imaging of an MMTV-PymT mouse after i.v. injection of DiD-labeled monocytes. Top row, left to right: pre-injection, 1-hr, 2–hr post-injection. Bottom row, left to right: 6-hr, 24-hr post-injection. **D.** Optical imaging of explanted left axillary tumor from the same mouse. Left to right: photograph only, fluorescence image. Images are representative of 6 MMTV-PymT mice injected with DiD-labeled monocytes.

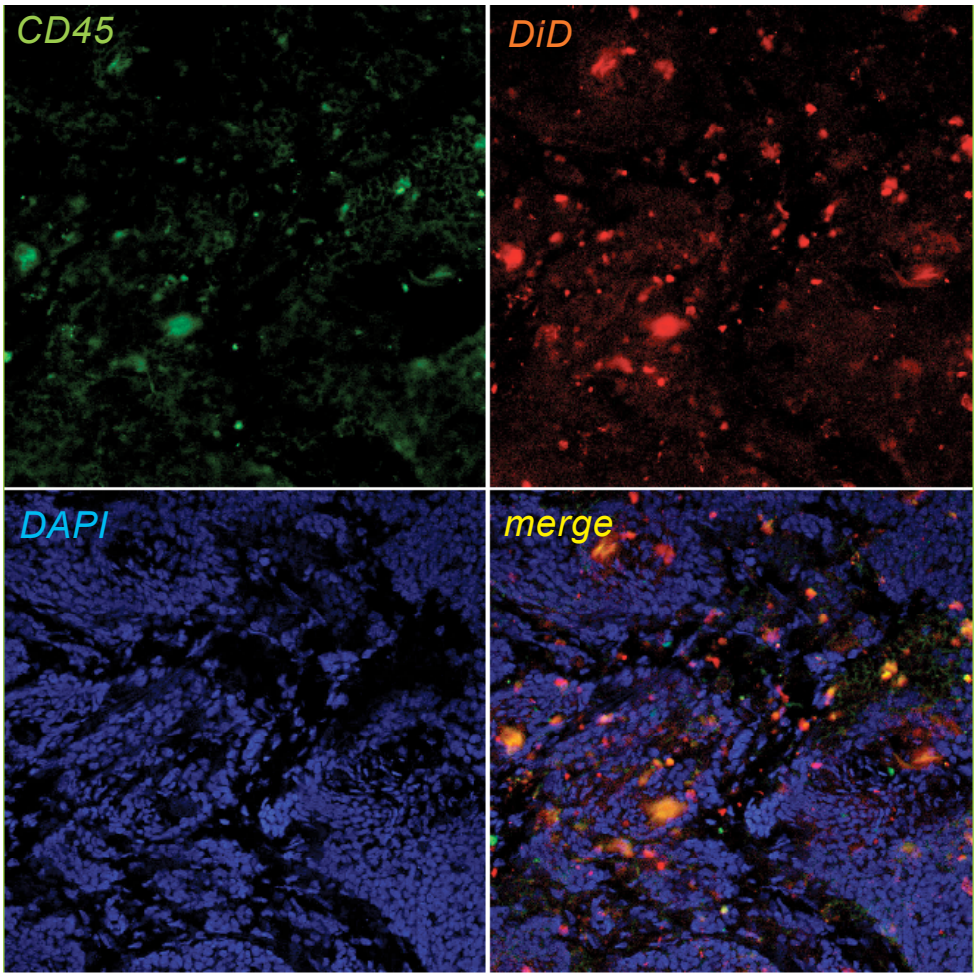


FIGURE 12: Immunofluorescent/confocal microscopy of DiD-labeled monocytes in PymT mammary tumor. Photomicrographs show CD45 (green staining), DiD labeled monocytes (red staining), DAPI nuclear stain (blue) and merge (orange) revealing the relative presence of DiD-labelled monocytes as compared to unlabelled CD45+ leukocytes.

III. KEY RESEARCH ACCOMPLISHMENTS:

Task 1. Define the profile and proteolytic contribution of leukocytes in human breast cancer and in transgenic mouse models of mammary carcinogenesis.

a. Elucidate the spectrum of CD45$^+$ cells in normal and neoplastic human breast tissues.

Months 1-12

- Acquired representative paraffin-embedded samples of human mammary tissue reflecting disease-free, ductal carcinoma in situ, and frank carcinoma (10 each)
- Performed histochemical evaluation by hematoxylin and eosin staining to affirm tissue and disease stage
- Performed Immunodetection analysis on tissue sections to reveal leukocyte infiltrates, e.g., CD45, CD4, CD8, CD68, neutrophil elastase, mast cell chymase, and CD31 for endothelial cells

Months 12-24:

- Initiated collaboration with Drs Susan Love and Lisa Bailey to acquire freshly resected breast cancer tissue and adjacent normal control tissue to utilize for FACS analysis

b. Elucidate the spectrum of CD45$^+$ cells in normal and neoplastic mammary tissues from mouse models of mammary carcinogenesis.

Months 1-24

- Generated breeding colony of MMTV-PymT mice and aged out to representative endpoints to collect tissues reflecting early mammary intraepithelial neoplasia (MIN), DCIS and carcinoma
- Performed histochemical evaluation by hematoxylin and eosin staining to affirm tissue and disease stage
- Performed Immunodetection analysis on tissue sections to reveal leukocyte infiltrates, e.g., CD45, CD4, CD8, CD68 and F4/80 for macrophages, 7/4 and CD11b for myeloid cells, CD119 for mast cells, and CD31 for endothelial cells
- Performed flow cytometric analyses on staged tissue suspensions from MMTV-PymT mice to quantitatively evaluate immune cell infiltrates at each stage of neoplastic progression.

Months 12-24

- Extended FACS analysis to include examination of leukocytes populations in blood and spleen in tumor bearing mice MMTV-PymT mice
- Performed histochemical evaluation by hematoxylin and eosin staining to affirm tissue and disease stage in PymT/RAG1$^{-/-}$, PymT/JH$^{-/-}$, PymT/CD4$^{-/-}$/CD8$^{-/-}$, PymT/CD4$^{-/-}$, PymT/CD8$^{-/-}$ mice
- Performed Immunodetection analysis on tissue sections to reveal leukocyte infiltrates, e.g., CD45, CD4, CD8, CD68 and F4/80 for macrophages, 7/4 and CD11b for myeloid cells, CD119 for mast cells, and CD31 for endothelial cells in PymT/RAG1$^{-/-}$, PymT/JH$^{-/-}$, PymT/CD4$^{-/-}$/CD8$^{-/-}$, PymT/CD4$^{-/-}$, PymT/CD8$^{-/-}$ mice
- Performed flow cytometric analyses on staged tissue suspensions from PymT/RAG1$^{-/-}$, PymT/JH$^{-/-}$, PymT/CD4$^{-/-}$/CD8$^{-/-}$, PymT/CD4$^{-/-}$, PymT/CD8$^{-/-}$ mice to quantitatively evaluate immune cell infiltrates at each stage of neoplastic progression

c. Develop a profile of proteolytic activities in normal and neoplastic human breast tissues.

Months 1-12

- Collaborated with the Sloane BCCOE to evaluate mRNA isolated human mammary tissue reflecting disease-free tissue, DCIS and Stage I, II and II disease on the Hu.Mu protease chip array

d. Develop a profile of proteolytic activities in normal and neoplastic mammary tissues from mouse models of mammary carcinogenesis.

Months 1-24

- Collaborated with Prof. Dylan Edwards to evaluate mRNA expression for cysteine cathepsin gene family members on staged tissues from MMTV-PymT mice

e. Determine cellular origins of proteolytic activities in normal and neoplastic human breast tissues.

Months 1-24

- These studies have not yet been initiated

f. Determine cellular origins of proteolytic activities in normal and neoplastic mammary tissues from mouse models of mammary carcinogenesis.

Months 1-24

- Evaluated origin of cathepsin C expression in MMTV-PymT tissue sections
- Used double Immunoflorescence staining to localize cathepsin C expression to infiltrating F4/80+ myeloid cells in metastatic lungs of PymT mice

g. Compare immune cell infiltrations in mouse and human normal and neoplastic tissues. Identify candidate cell types for further study.

Months 1-24

- These comparative studies have not yet been initiated

h. Compare proteolytic activities in mouse and human normal and neoplastic tissues based. Identify candidate protease activities for further study.

Months 1-24

- These comparative studies have not yet been initiated

Task 2. Validate target molecules and/or specific immune cell types in biological assays and in animal models of mammary carcinogenesis.

a. Establish 3-dimensional mammary epithelial organotypic cell culture model system cultured on basement membrane.

Months 1-9

- Dr. DeNardo was trained in development, isolation, culture and maintenance of 3D organotypic cultures by Dr. Jay Debnath (UCSF).
- Routine generation, culture and maintenance of 3D organotypic cultures established in Coussens.
- Routine isolation of primary MECs from wt and MMTV-PymT mice established in Coussens lab and subsequent culture in 3D routine.

b. Establish routine isolation and short-term culture conditions for mouse leukocytes.

Months 1-9

- Routine isolation and culture of primary macrophages, T cells, immature myeloid cells, macrophages and mast cells established in Coussens lab from peripheral blood, spleen, wt mammary glands and early and late-stage carcinomas from MMTV-PymT mice.

c. Establish 3-dimensional organotypic co-culture model system for analysis of paracrine interactions between mammary epithelial cells and specific leukocytic cell types.

Months 6-12

- 3D co-culture of nMECs and pMECs with naïve and tumor-associated macrophages established in Coussens lab
- 3D co-culture of nMECs and pMECs with naïve and tumor-associated macrophages and CD4+ T cells established in Coussens lab

d. Establish 3-dimensional organotypic culture model system for analysis of paracrine interactions between mammary epithelial cells and specific leukocytic-derived proteases.

Months 6-12

- Paracrine regulation of MECs by T cells and macrophages identified as being metalloproteinase-dependent

e. Analyze role of leukocytes in regulating mammary epithelial morphogenesis, proliferation, cell death, apicobasal polarity, cell-cell adhesion, and formation of invasive and protrusive structures in normal and

oncogene-expressing 3-dimensional acini cultured on basement membrane.

Months 12-24

- Acinus disruption assay validated in Coussens lab
- Paracrine regulation of MECs by CD4+ T cells and macrophages identified as IL4-dependent
- Paracrine regulation of MECs by CD4+ T cells and macrophages identified as utilizing Shh and Wnt proteins
- Paracrine regulation of MECs by CD4+ T cells and macrophages is due to differential activation of T_H2-type cytokines versus repression of T_H1-type cytokines
- Paracrine regulation of MECs by CD4+ T cells and macrophages involves increased expression of EGF

f. Analyze role of leukocyte-derived proteases in regulating mammary epithelial morphogenesis, proliferation, cell death, apicobasal polarity, cell-cell adhesion, and formation of invasive and protrusive structures in normal and oncogene-expressing 3-dimensional acini cultured on basement membrane.

Months 12-36

- These studies have not been initiated

g. Analyze role of candidate leukocytes by crossing mice deficient in or modified such that individual or classes of leukocytes (identified in Task 1) are deficient with transgenic mice prone to development of mammary adenocarcinoma.

Months 1-12

- MMTV-PymT mice intercrossed with $RAG1^{-/-}$ mice
- MMTV-PymT mice intercrossed with $CD4^{-/-}CD8^{-/-}$ mice
- MMTV-PymT mice intercrossed with $JH^{-/-}$ mice
- Evaluate primary tumor and pulmonary metastasis development in crosses
- T cells identified as significant adaptive leukocyte regulating pulmonary metastasis formation

Months 12-24

- MMTV-PymT mice intercrossed with $CD4^{-/-}$ mice
- MMTV-PymT mice intercrossed with $CD8^{-/-}$ mice
- Assessment of neoplastic progression in PymT/$RAG1^{-/-}$, PymT/$JH^{-/-}$, PymT/$CD4^{-/-}$/$CD8^{-/-}$, PymT/$CD4^{-/-}$, PymT/$CD8^{-/-}$ mice
- Metastasis in PymT mice found to be CD4+ T cell-dependent
- CD4+ T cells found to repress TH1-type cytokine expression by tumor-associated macrophages
- CD4+ T cells found to induce TH2-type cytokine expression by tumor-associated macrophages

h. Analyze role of proteases expressed by both tumor and stromal cells by crossing transgenic mice deficient in a protease already implicated in breast cancer progression with transgenic mice prone to development of mammary adenocarcinoma

Months 1-12

- MMTV-PymT mice intercrossed with cathepsin $C^{-/-}$ mice

i. Analyze role of proteases expressed by leukocytes by crossing transgenic mice deficient in a candidate leukocyte identified in Task 1 with transgenic mice prone to development of mammary adenocarcinoma.

Months 12-24

- Breeding colonies of PymT/cathepsin C +/- and -/- mice generated, expanded and aged out to endpoints
- PymT/cathepsin C -/- mice found to have significantly reduced incidence of pulmonary metastasis
- Cohorts of PymT/cathepsin C +/- are still aging out for completion
- Cathepsin C found to be a significant regulator of pulmonary metastasis formation

Task 3. Develop non-invasive imaging reagents to monitor leukocyte and/or protease-specific events during mammary carcinogenesis

a. Identify and characterize selective peptide substrates and selective binding peptides for proteases already implicated in breast cancer progression.

Months 1-24

- RDM designed using MMP14-cleavage peptides
- RDM biochemically assessed for selective MMP-14 cleavage

b. Identify and characterize selective peptide substrates and selective binding peptides for candidate proteases validated in Task 2.

Months 24-48

- Initiate re-synthesis of FY01 cathepsin C activity probe with fluorescent tag for ex vivo and in vivo analyses

c. Synthesize novel fluorescent probes for imaging protease activity using peptide substrates identified above (3.a.).

d. Demonstrate ability of fluorescently-labeled molecules to localize to xenograft and/or 3-dimensional organotypic cancer models using confocal fluorescence and/or whole-body fluorescence imaging.

Months 6-12

- RDM optically screened in cell-based cultures
- Selective uptake of RDM affirmed in cell-based analyses
- Bodipy-RDM probe evaluated in murine breast cancer xenograft and selectivity analyses initiated

Months 12-24

- Monocytes labeled with DiD
- In vitro optical imaging validated of monocytes validated
- FACS analysis of labeled monocytes to confirm cell surface marker expression

e. Identify and characterize covalently coated iron oxide nanoparticles with poly(ethylene glycol) polymers functionalized with specific peptides or antibodies that uniquely interact with target immune cell populations.

Months 1-12

- Not initiated in months 1-12

Months 12-24

- Not initiated in months 12-24

f. Validate covalently coated iron oxide nanoparticles with poly(ethylene glycol) polymers functionalized with specific peptides or antibodies for selectivity in the organotypic 3-dimensional co-culture models.

Months 12-48

- Not initiated in months 1-24

g. Validated covalently coated iron oxide nanoparticles with poly(ethylene glycol) polymers functionalized with specific peptides or antibodies in vivo in the parental, immune and/or protease-modified mouse models of mammary carcinogenesis. Confirm relationship between level of nanoparticle uptake within tissues and level of immune cell infiltration histologically.

Months 1-12

- Not initiated in months 1-12

Months 12-24

- Optical imaging of DiD-labeled monocytes in MMTV-PymT and control mice
- Confocal microscopy of tissue sections to confirm presence of DiD-labeled monocytes in mammary tumors
- Quantitative analysis of fluorescence from mammary tumors following injection of DiD-labeled monocytes

h. Analyze evolution of leukocyte infiltration and/or protease expression in mouse mammary models using fluorescently-labeled molecular probes where animals are imaged longitudinally on weekly intervals.

Months 12-60.

- Not initiated in months 1-24

i. Select candidate molecular probes emerging from above (3.d.) that demonstrate the capability of being detected in vivo and are present at key timepoints in the evolution of breast cancer and use as a platform for development of protease-specific radiolabeled probes for single photon emission computed tomography (SPECT).

Months 12-60

- Not initiated in months 1-24

j. Demonstrate ability of agents in 3.i. to localize to tumors in proportion to the level of the specific protease targeted using in-vivo SPECT-CT imaging (X-SPECT, Xenogen Corp.) with ex-vivo autoradiography, scintillation well-counting, and immunohistochemistry to pathologically confirm levels of tissue expression and/or immune cell infiltration in areas concentrating the radiolabeled probe.

Months 24-60.

- Not initiated in months 1-24

k. Monitor relationships between presence of various immune cell populations and protease expression within mouse mammary models utilizing multi-modality imaging (MRI, SPECT-CT) in combination with the immune cell (MR) agents and protease (SPECT-CT) agents developed in 3.e. and 3.i., respectively.

Months 36-60.

- Not initiated in months 1-24

IV. REPORTABLE OUTCOMES:

A. MANUSCRIPTS: (PROVIDED AS APPENDIX MATERIAL B-D)

Months 1-12 (Provided as Appendix Material in 2007 Annual Progress Report)

Tan TT, **Coussens LM**. Humoral immunity, inflammation and cancer. (2007) *Curr Opin Immunology* 19(2), 209-216

Johansson M, Tan T, de Visser KE, **Coussens LM**. (2007) Immune cells as anti-cancer therapeutic targets and tools. *J Cellular Biochemistry*, *in press. J Cellular Biochemistry*, 101: 918-926.

DeNardo D, **Coussens LM**. (2007) Balancing immune response: Crosstalk between adaptive and innate immune cells during breast cancer progression. *Breast Cancer Research,* 9:212-222.

Months 12-24 (Provided as Appendix Material E-H)

Eichten AE, Hyun WC, **Coussens LM**. (2007) Distinctive features of angiogenesis and lymphangiogenesis determine their functionality during de novo tumor development. *Cancer Research,* 67:5211-5220.

Kenny H, Kaur S, **Coussens LM**, Lengyel E. (2008) Adhesion of OvCa cells to peritoneum is mediated by MMP-2 cleavage of fibronectin*, J Clin Invest,* 118(4):1367-1379.

DeNardo DG, Johansson M, **Coussens LM**. (2008) Immune cells as mediators of solid tumor metastasis. *Cancer Metastasis Rev*. 27:11-18.

Johansson M, DeNardo DG, **Coussens LM**. (2008) Polarized immune responses differentially regulate cancer development. Immunol Rev., 222:145-154.

B. ABSTRACTS: (COMPILED AND PROVIDED AS APPENDIX I)

Months 1-12

Coussens LM., Microenvironmental Regulation of Vascular Homeostasis and Leakage, AACR Mouse Models of Cancer, Cambridge, MA, USA

Coussens LM., Inflammation, Proteases and Cancer Development, International Society for Oncodevelopmental Biology and Medicine (ISOBM) Meeting, Pasadena, CA, USA

Coussens LM., Immune Cells as Targets for Cancer Prevention, 18th Annual Pezcoller Symposium, Trento, ITALY

Coussens LM., Inflammation, Proteolysis and Cancer Development, 37th International Symposium of the Princess Takamatsu Cancer Research Fund, Tokyo, JAPAN

Coussens LM., Inflammation, Proteolysis and Cancer Development, 2007 AAAS Annual Meeting, San Francisco, CA USA

Coussens LM., Inflammation, Proteolysis and Cancer Development, 7th AACR-Japanese Cancer Association Joint Conference, Waikaloa, Hawaii, USA

Coussens LM., Inflammation, Humoral Immunity and Epithelial Cancer Development, The Second International Symposium on Cancer Metastasis and the Lymphovascular System: Rational Basis for Therapy, San Francisco CA USA

Coussens LM., Inflammation, Humoral Immunity and Epithelial Cancer Development, 2007 Keystone Symposium on Mechanisms Linking Inflammation and Cancer, Santa Fe, New Mexico. USA

Coussens LM., Inflammation, Humoral Immunity and Cancer Development, International Cancer Microenvironment Society (ICMS) - American Association of Cancer Research, 4th International Conference on Tumor microenvironment. Florence ITALY

DeNardo D, Baretto JB and Coussens LM. *Macrophages Regulate Mammary Epithelial Ducal Polarity,* 2006 AACR Workshop in Cancer Research: Pathology of Cancer. Snowmass. CO

DeNardo D, Baretto J, Coussens LM. Macrophages regulate mammary epithelial ductal polarity. 2007 Keystone Symposium on Mechanisms Linking Inflammation and Cancer, Santa Fe, New Mexico. USA

Months 12-24

Coussens LM., Inflammation and Cancer: Organ-specific Regulation of Cancer Development, 2007 AACR, *Frontiers in Cancer Prevention Research Conference*, Philadelphia, PA, USA

Coussens LM., Inflammation, Humoral Immunity and Epithelial Cancer Development, 2007 , *7th International Symposium on Hodgkin Lymphoma*, Cologne, GERMANY

Coussens LM., Immune Modulation of Epithelial Cancer Development, 2007 22nd Aspen Cancer Conference: Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention and Cancer Therapy, Aspen CO, USA

Coussens LM., Inflammation and Cancer: Organ-specific Regulation of Cancer Development, 2008 47th Midwinter Conference of Immunologists, '*Meeting the challenge: Immunobiology in health and disease*', Asilomar, CA USA

DeNardo D, Baretto J, Coussens LM. 2007 Timberline Symposium on: 3D tissue Biology: Human Stem Cells, Cancer and the Microenvironment. Portland, OR.

Coussens LM., Inflammation and Cancer: Organ-specific Regulation of Cancer Development, 2008 Keystone Joint Symposium, '*Cell Death in the Immune System / Cell Death and Cellular Senescence'*, Beaver Run Resort in Breckenridge, CO, USA

Coussens LM., Inflammation and Cancer: Organ-specific Regulation of Cancer Development, 2008 Keystone Symposium, '*Inflammation, Microenvironment and Cancer'*, Snowbird Resort in Snowbird, Utah, USA

DeNardo D, Baretto J, Coussens LM. 2008. Midwinter Conference of Immunologists. Asilomar Ca

DeNardo D, Baretto J, Coussens LM. 2008 International Conference of Invasion and Metastasis, Berlin, Germany.

DeNardo D, Baretto J, Coussens LM. AACR Annual Meeting, San Diego, CA,

C. PRESENTATIONS (COUSSENS, LISA M):

Symposia and Workshops: **International**

2006 18th Annual Pezcoller Symposium *'Tumor Microenvironment: Heterotypic Interactions'*, Trento ITALY

2006 European Association for Cancer Research (EACR) 1st Annual Meeting, Budapest HUNGARY

2006 XXXIVth Meeting of the International Society for Oncodevelopmental Biology and Medicine (ISOBM: Tumor Biology, Detection and Therapy, Pasadena, CA, USA

2006 37th International Symposium of the Princess Takamatsu Cancer Research Fund '*Cancer Cells and Their Microenvironment*', Tokyo, JAPAN

2007 *4th International Conference on Tumor Microenvironment*, Florence, ITALY

2007 *2nd International Symposium on Cancer Metastasis and the Lymphovascular System*: Basis for Rational Therapy, San Francisco CA USA

2007 CNIO – Nature Symposium on "*Oncogenes and Human Cancer*". The Next 25 Years", Madrid SPAIN

2007 KEYNOTE LECTURE, *7th International Symposium on Hodgkin Lymphoma*, Cologne, GERMANY
2007 CANDLELIGHT LECTURE, *Inflammation and Cancer: From molecular links to bed side*; Inaugural meeting for the *Istituto Clinico Humanitas*, Milan ITALY

Symposia and Workshops: **National**
2006 Genetic, Cellular and Microenvironmental Determinants of Tumor Progression and Metastasis: A 'TPM' Workshop Honoring Martin L Padarathsingh, Ph.D. TPM Study Section Workshop, Natl. Cancer Institute, Georgetown, VA, USA
2006 ASCO/Federation of European Societies Symposium: *Inflammation in Cancer Progression*, 2006 ASCO Annual Meeting, Atlanta, GA, USA
2006 AACR Special Conference, *Mouse Models of Cancer*, Cambridge, MA, USA
2006 AACR Special Conference, *Tumor Immunology: An Integrated Perspective.* Miami, FL, USA
2007 7th AACR-Japanese Cancer Association Joint Conference: *In the Forefront of Basic and Translational Cancer Research*, Waikoloa, Hawaii, USA
2007 Keystone Symposium, *'Mouse Models at the Frontiers of Cancer Discovery'*, Whistler, British Columbia, CANADA
2007 Keystone Symposium '*Inflammation and Cancer'*, Santa Fe, NM, USA
2007 AAAS Annual Meeting, *Healthy Aging: Inflammation and Chronic Diseases'* Symposium, San Francisco, CA USA
2007 Tumor Microenvironment and Tumor-Stromal Interactions Workshop: Sponsored by Biogen Idec Inc., Oncology Discovery Research, San Diego CA USA
2007 American Thoracic Society 2007 International Conference, *San Francisco Science: Inflammation, Immunity and Signaling*. San Francisco, CA USA
2007 22nd Aspen Cancer Conference: Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention and Cancer Therapy, Aspen CO, USA
2007 Gordon Research Conference, *Epithelial Differentiation & Keratinization*, Bryant University, Smithfield, RI, USA
2007 AACR, *Frontiers in Cancer Prevention Research Conference*, Philadelphia, PA, USA
2007 National Cancer Institute Workshop, '*Profiling of Immune Response to Guide Cancer Diagnosis, Prognosis and Prediction of Therapy'*, Bethesda, MD, USA
2008 47th Midwinter Conference of Immunologists, '*Meeting the challenge: Immunobiology in health and disease*', Asilomar, CA USA
2008 AACR-TREC-NCI Conference on *Energy Balance and Cancer: Mediators and Mechanisms*, Lansdowne, VA USA
2008 Keystone Joint Symposium, '*Cell Death in the Immune System / Cell Death and Cellular Senescence'*, Beaver Run Resort in Breckenridge, CO, USA
2008 Keystone Symposium, '*Inflammation, Microenvironment and Cancer'*, Snowbird Resort in Snowbird, Utah, USA
2008 The John F. Anderson Memorial Lectures in Medicine, '*The Linkage between Inflammation and Cancer*', University of Virginia, Charlottesville VA, USA
2008 *Tumor Microenvironment Symposium*, Stony Brook University, Stony Brook. NY. USA
2008 KEYNOTE LECTURE, Fox Chase Cancer Center 13th Annual Postdoctoral Fellow and Graduate Student Symposium, Philadelphia, PA USA

Invited Lectures/Seminars: International
2007 Angiogenesis and Tumor Targeting Research Unit & Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, ITALY
2008 Institute of Cell Biology, ETH Zurich Switzerland

Invited Lectures/Seminars: National
2007 *Lymphoma and Myeloma Conference*, M.D. Anderson Cancer Center, Houston, TX, USA
2007 University of Minnesota, Dept of Lab Medicine and Pathology, Minneapolis, MN, USA
2007 Memorial-Sloan Kettering Cancer Center, Program in Cancer Biology and Aging, New York NY, USA

2007 Abramson Family Cancer Research Institute and Univ. of Pennsylvania, Division of Hematology-Oncology, Philadelphia, PA USA
2007 Albert Einstein College of Medicine, New York NY, USA
2007 Oncology Division Research, Biogen Idec Inc., San Diego, CA USA
2007 Genentech, Inc. Immunology Program. South San Francisco, CA USA
2007 University of Iowa Carver College of Medicine, Dept of Pathology, *Pathology Grand Rounds*, Iowa City, Iowa, USA
2007 Hollings Cancer Center, Medical University of South Carolina, Charleston, SC, USA
2007 University of Michigan, Program in Immunology and Cancer Research Series, Ann Arbor, MI USA
2008 Department of Pathology/UCLA School of Medicine Seminar, Los Angeles CA USA
2008 ANNUAL KEYNOTE LECTURE, Dept of Cancer Biology, Meharry Medical College, Nashville, TN USA
2008 University of California, Davis Cancer Center, Sacramento, CA USA
2008 Department of Immunology, University of Pittsburgh School of Medicine. Pittsburgh, PA, USA
2008 Cancer Biology Series, Ben May Cancer Center, University of Chicago, Chicago, IL, USA

Presentations (Coussens Lab members):
2007 **DeNardo DG**, Innate and adaptive immune cell regulation of mammary carcinogenesis. UCSF Programs in Tumor Immunity and Hematopoietic malignancies, June 2007.
2008 **DeNardo DG**, AACR Annual Meeting, San Diego, CA,
2008 **DeNardo DG**, International Conference of Invasion and Metastasis, Berlin, Germany
2007 **DeNardo DG**, Timberline Symposium on: 3D tissue Biology: Human Stem Cells, Cancer and the Microenvironment. Portland, OR
2007 **DeNardo DG**, University of California Davis, Symposium on Emerging Challenges in Microbiology and Immunology. Davis, CA.

D. PATENTS AND LICENSES: N/A

E. DEGREES OBTAINED: N/A

F. REAGENT DEVELOPMENT:
- Generation of breeding colony of MMTV-PymT mice on the FVB/n strain background
- Generation of breeding colony of MMTV-neu mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/RAG1$^{-/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/CD4$^{-/-}$/CD8$^{-/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/JH$^{-/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/cathepsin C$^{-/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/CD4$^{-/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/CD8$^{-/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/RAG1$^{+/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/CD4$^{+/-}$/CD8$^{+/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/JH$^{+/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/cathepsin C$^{+/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/CD4$^{+/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/CD8$^{+/-}$ mice on the FVB/n strain background
- Generation of primary mammary adenocarcinoma cell line established from day 95 MMTV-PymT (FVB/n) mouse, i.e., Min#1
- Generation of primary mammary adenocarcinoma cell line established from day 76 MMTV-PymT (FVB/n) mouse, i.e., Min#2
- Generation of primary mammary adenocarcinoma cell line established from day 110 MMTV-PymT (FVB/n) mouse, i.e., O1-T1
- Generation of pulmonary metastasis of mammary adenocarcinoma cell lines established from day 110 MMTV-PymT (FVB/n) mouse, i.e., MET#2, #3, and #4

- Generation of pcHRED transfected stable subclone of MET#2 mammary adenocarcinoma pulmonary metastasis cell line established from day 95 MMTV-PymT (FVB/n) mouse, i.e., MET#2-pHcRED#1, MET#2-pHcRED#2, MET#2-pHcRED#3
- Generation of a mouse cathepsin C expression vector by insertion of murine cathepsin C cDNA into pCR2.1 vector

G. FUNDING APPLIED FOR BASED ON WORK SUPPORTED BY ERA OF HOPE:

DeNardo DG, 2006. American Cancer Society Postdoctoral fellowship
LOVE S AND **COUSSENS LM**., 2008, CDMP/DOD, Synergistic Idea Award

H. EMPLOYMENT/RESEARCH OPPORTUNITIES APPLIED FOR: N/A

V. CONCLUSION

Chronic inflammation, a pathological condition resulting from enhanced and sustained migration of leukocytes into tissue, is now regarded as a promoting force in the majority of all solid tumors in humans. When leukocytes migrate into damaged tissues, they produce a variety of soluble mediators, including growth factors and numerous proteases that promote cancer by providing growth and survival factors to initiated neoplastic cells, regulate proangiogenic programs. Moreover, it is now quite well accepted that subpopulations of these same leukocytes also possess potent proangiogenic activity as well as immune suppressive capabilities that block effective anti-tumor T cell responses. Thus, identification of the molecular and cellular pathways that regulate these two distinct pro-tumor bioactivities would reveal identification of molecules or pathway that could be targeted to neutralize the pro-growth properties of activated leukocytes, as well as those that suppress effective anti-tumor T cell immunity. Because tumor-associated myeloid cells are genetically stable cells, they are less likely to develop drug resistance than cancer cells, and drugs that inhibit selected hematopoietic cell functions should hold promise for effective anti-cancer treatments. Established tumors represent formidable opponents that harbor inherent potential for developing drug resistance. Aside from investing in earlier screening to detect and eradicate premalignant disease, our best hope for minimizing cancer is to develop combinatorial treatment strategies where intrinsic pathways regulating neoplastic cell survival are targeted, in combination with therapies effecting extrinsic pathways that neutralize pro-tumor immunity, bolster anti-tumor immunity and limit or normalize angiogenic blood vessels. Our belief is that a broader understanding of immune cells and the specific proteolytic molecules they express during will lead to development of novel anti-cancer treatments.

Previous studies from the Coussens laboratory have demonstrated that inhibition of leukocyte migration and/or leukocyte-derived proteases into hyperplastic tissue during skin carcinogenesis is sufficient to significantly decrease tumor incidence; thus, indicating that leukocyte recruitment is a functionally significant parameter of cancer development and that inflammation may be targeted pharmacologically to affect outcome. While we have only been experimentally addressing similar questions regarding breast carcinogenesis utilizing *in vivo* mouse and *in vitro* organotypic models for only two-years now, our compelling preliminary data indicate that indeed, mammary carcinogenesis is similarly susceptible to immuno-modulation as a therapeutic modality. We have revealed that infiltrating CD4+ T cells alter the primary mammary microenvironment in such a way that pulmonary metastasis is favored. Our data thus far indicate that infiltrating CD4+ T cells are heavily T_H2 polarized, and via their secretion of IL-4, regulate macrophage effector function that enables MECs to exit the primary tumor microenvironment and metastasize to pulmonary locales. While we do not yet know how cathepsin C plays into this response, the diminished number of pulmonary metastases that form in cathepsin C-deficient mice emanating from significantly fewer circulating malignant MECs indicates that cathepsin C is likely exerting its role also within the primary tumor microenvironment. In the next funding period, we will evaluate how CD4 T cells and cathepsin C modulate the primary tumor microenvironment and/or the microenvironment in lungs to affect metastasis. The organotypic cultures will be informative here as we can manipulate them rapidly to reveal mechanisms involved. Together, these studies will provide insight into the role adaptive immune cells play and how a leukocyte protease, e.g., cathepsin C, together regulate cancer development that will reveal a potential novel mechanism with which to target tumor cells with anti-cancer therapeutics. While our novel SPECT RDA molecular probes have been validated in vivo their likely translation

to a clinical tool is limited; thus, we have initiated studies to evaluate if primary monocytes can be utilized as noninvasive imaging reagents. Our preliminary studies indicate that this is potentially a viable approach using primary monocytes labeled with DiD followed by whole body optical image analysis. We will pursue this route of investigation to reveal if CD4 T cells and/or selective populations of myeloid cells can similarly be labeled and imaged noninvasively following their appropriate trafficking back to either primary or metastatic tumors. Once these studies are validated, we will evaluate quantum dot nanocrystal-labeled monocytes as an additional reagent to pursue.

VI. BIBLIOGRPHY:

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VII. APPENDICES

A. Complete academic Curriculum vitae for Dr. Lisa M. Coussens

B. Tan TT, **Coussens LM**. Humoral immunity, inflammation and cancer. (2007) *Curr Opin Immunology* 19(2), 209-216

C. Johansson M, Tan T, de Visser KE, **Coussens LM**. (2007) Immune cells as anti-cancer therapeutic targets and tools. *J Cellular Biochemistry*, *J Cellular Biochemistry*, 101: 918-926.

D. DeNardo D, **Coussens LM**. (2007) Balancing immune response: Crosstalk between adaptive and innateimmune cells during breast cancer progression. *Breast Cancer Research,* 9:212-222.

E. Eichten AE, Hyun WC, **Coussens LM**. (2007) Distinctive features of angiogenesis and lymphangiogenesis determine their functionality during de novo tumor development. *Cancer Research,* 67:5211-5220.

F. Kenny H, Kaur S, **Coussens LM**, Lengyel E. (2008) Adhesion of OvCa cells to peritoneum is mediated by MMP-2 cleavage of fibronectin*, J Clin Invest,* 118(4):1367-1379.

G. DeNardo DG, Johansson M, **Coussens LM**. (2008) Immune cells as mediators of solid tumor metastasis. *Cancer Metastasis Rev*. 27:11-18.

H. Johansson M, DeNardo DG, **Coussens LM**. (2008) Polarized immune responses differentially regulate cancer development. Immunol Rev., 222:145-154.

I. Collected Abstracts from Months 1-24

APPENDIX A

CURRICULUM VITAE

June 2008

Lisa M. Coussens, Ph.D.
Professor
Department of Pathology and Cancer Research Institute
University of California, San Francisco
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http://cancer.ucsf.edu/coussens/index.php

I. EDUCATION:

1976 - 1980	San Francisco State University	B.A.	Biology
1988 - 1993	University of California, Los Angeles	Ph.D.	Biological Chemistry
1993 - 1997	University of California, San Francisco	Post-Doctoral Fellow	Cancer Biology

II. PRINCIPAL POSITIONS HELD:

1981 - 1988	Genentech, Inc., South San Francisco	Research Associate	Molec. & Devel. Biology
1997 - 1999	University of California, San Francisco	Assistant Research Biochemist	Hormone Research Inst.
1999 - 2004	University of California, San Francisco	Assistant Professor, In Residence	Cancer Research Inst. & Dept of Pathology
2004 - 2006	University of California, San Francisco	Associate Professor, In Residence	Cancer Research Inst. & Dept. of Pathology
2006 - 2007	University of California, San Francisco	Associate Professor	Dept. of Pathology & Cancer Research Inst.
2007 - present	University of California, San Francisco	Professor	Dept. of Pathology & Cancer Research Inst.

OTHER POSITIONS HELD CONCURRENTLY:

1989 - 1992	Whittier College, Whittier, CA	Lecturer	Biology Dept
1992	Genentech, Inc., South San Francisco	Scientific Consultant	Dept. of Legal Affairs

III. HONORS AND AWARDS:

1985	Recognition Award	Genentech, Inc.,
1986	Recognition Award	Genentech, Inc.,
1988	Recognition Award	Genentech, Inc.
2000 - 02	Hellman Family Award For Early Career Faculty	Univ. of Calif., San Francisco
2000 - 01	V Foundation Scholar	The V Fndt. for Cancer Research
2000 - 03	Edward Mallinckrodt, Jr. Fndt. Award for Medical Research	Edward Mallinckrodt, Jr. Fndt.
2002	Gertrude B. Elion Cancer Research Award	Am. Assoc. for Cancer Research
2006 - 11	Era of Hope Scholar Award	Department of Defense, Breast Cancer Research Program

IV. PROFESSIONAL ACTIVITIES

1999 - present	Member	Graduate Program in BioMedical Sciences (BMS)	Univ. of Calif., San Francisco

1999 - present	Member	Helen Diller Family Comprehensive Cancer Center	Univ. of Calif., San Francisco
2000 - present	Member	Graduate Program in Biological Sciences (PIBS)	Univ. of Calif., San Francisco
2001 - present	Co-Director	Mouse Pathology Core	Helen Diller Family Comprehensive Cancer Center, Univ. of Calif., San Francisco
2004 – present	Member	Program in Immunology	Univ. of Calif., San Francisco
2004 – 2007	Senior Editor	***Cancer Research (****Cell, Tumor and Stem Cell Biology* Section)	American Association Cancer Research
2007 – 2009	Deputy Editor	***Cancer Research***	American Association Cancer Research
2007 – 2009	Senior Editor	***Cancer Research (****Tumor Microenvironment* Section)	American Association Cancer Research
2007 - 2010	Member	External Scientific Advisory Board, University of Minnesota Cancer Center	University of Minnesota Cancer Center
2007 – 2011	Member	External Scientific Advisory Board, CA U54: *Aging, Tumor Microenvironment and Prostate Cancer*; P.I. Steve Plymate, Univ. of Washington, HMC.	University of Washington
2008 – 2011	Member	Board of Directors (*elected*)	American Association of Cancer Research

V. PROFESSIONAL ORGANIZATIONS

Memberships

2000 - present	American Society for Matrix Biology
2000 - present	American Association for Cancer Research
2001 - present	American Society for Cell Biology
2004 - present	American Society for Investigative Pathology
2004 - present	International Protease Society

Service to Professional Organizations

American Association for Cancer Research

2003	Subsection Co-chair (Tumor Progression, Invasion and Metastasis) Cellular, Molecular and Tumor Biology Subcommittee, AACR Program Committee for *94th Annual Meeting*.
2003	Chair and organizer, Educational Session *(*Proteases: Successes and Failures): *94th Annual Meeting*, Washington D.C., USA
2003	Minisymposium Co-chair (Inflammatory Mediators & Cancer): *94th Annual Meeting*, Washington D.C., USA
2004 - 2006	Member, Grants Committee
2005	Minisymposium Co-Chair (Inflammation, Microenvironment and Tumor Progression): *96th Annual Meeting*, Anaheim, CA USA
2005	Session Chair (Inflammation*)*: *AACR Special Conference: Cancer, Proteases and the Microenvironment*, Bonita Springs, Florida. USA
2006	Subsection Co-chair (Tumor Progression, Invasion and Metastasis) of the Tumor Biology Subcommittee, AACR Program Committee for *97th* Annual meeting
2006	Minisymposium Co-Chair (Inflammation and Cancer): *97th Annual Meeting*, Washington DC, USA
2006	Co-Chairperson, Program Committee: *6th Annual Frontiers in Cancer Prevention Research Conference*, December 5-8, 2007, Philadelphia, PA USA.
2006-2009	Steering Committee Member: AACR Tumor Microenvironment Working Group (TME/AACR).

2007 Organizer, Education session (Inflammation and Cancer), *98th Annual Meeting*, Los Angeles, USA
2007 Minisymposium Co-Chair (Tumor Microenvironment): *98th Annual Meeting*, Los Angeles, CA USA
2007 Co-Chairperson, Program Committee: *2008 99th Annual Meeting of the AACR*. April 12-16, 2008, San Diego, CA. USA
2008 Program Committee Member, Tumor Microenvironment Subcommittee for *99th Annual Meeting of the AACR*. April 12-16, 2008, San Diego, CA. USA
2007-2010 Member, AACR Special Conferences Committee
2008 Co-Organizer Special Conference: *Inflammation and Cancer*, with Drs. Michael Karin and Larry Marnett. Oahu, Hawaii, USA.
2008-2011 Member, Board of Directors (elected)

American Society for Cell Biology
2000 American Society for Cell Biology, photo credits in '*Exploring the Cell'* Ed. W. Wells
2001 Table Leader, Career Discussion Lunch, Women in Cell Biology and Education Committee, 40th Annual Meeting, Washington, DC, USA
2001 Co-chair and Co-organizer, Mini-symposium (Microenvironment/Extracellular Matrix in Development and Disease): 40th Annual Meeting, Washington, DC, USA
2003 Table Leader, Career Discussion Lunch, Women in Cell Biology and Education Committee of the ASCB, *42nd Annual Meeting*, San Francisco, CA, USA
2006 Co-Chair Minisymposium (Cancer Mechanisms*)*: *46th Annual Meeting*, San Diego CA, USA

American Cancer Society
1999 14th Annual Excalibur Round Table, San Francisco, CA, USA
2000 San Mateo County Annual Volunteer Meeting, San Mateo, CA, USA

International Society for Preventive Oncology
2002 Session Chair (Chemoprevention*)*: *6th Annual Meeting*, Pasteur Institute, Paris, France.
2002 Poster Judge (Chemoprevention*)*: *6th Annual Meeting*, Pasteur Institute, Paris, France.

International Proteolysis Society
2007 Member, International Scientific Advisory Committee, 5th General Meeting of the International Proteolysis Society, Rion-Patras, GREECE.

International Society for Biological Therapy of Cancer
2008 Co-Organizer, *2008 Workshop on Inflammation in Cancer Development*, San Diego CA, USA

Service to Professional Publications:

2003 - 2005	Associate Editor, ***Cancer Research***
2005 – 2007	Editorial Board, ***Carcinogenesis***
2004 – 2007	Senior Editor, ***Cancer Research (****Cell, Tumor and Stem Cell Biology* Section)
2007 – present	Senior Editor, ***Cancer Research (****Tumor Microenvironment* Section)
2007 – present	Deputy Editor, ***Cancer Research***
2007	Guest Editor, PNAS Editorial Board
2008	Guest Editor (with Tyler Jacks), Current Opinion in Genetics & Development

Ad hoc reviewing
1994 Oncogene;
1995 Am J Pathology; Matrix Biology; J Cell Biology
1999 Am J Pathology; Cancer Letters; Nature Medicine; Nature; PNAS; Cell Motility & the Cytoskeleton; Cancer Research
2000 Am J Pathology; Cancer Research; Genes & Development; Int. J Cancer

2001 J Cell Biology; Int. J of Cancer; EMBO; Neoplasia; Cancer Research
2002 Cancer Research; Am J Pathology; Int. J Cancer; Biological Chemistry; Cancer Cell; Cancer Letters
2003 PNAS; Cancer Research; Int. J of Cancer; J Molecular Medicine; Biological Chemistry; Science; Cancer Cell; Nature Medicine; J Leukocyte Biology; Neoplasia; Am J Pathology
2004 Lancet; Cancer Cell; Cancer Research; American J Pathology; J Cell Biology; Nature Reviews Immunology; Nature Reviews Cancer; PNAS; J Biological Chemistry; Nature; J Exp Med; Int J Cancer
2005 Nature Medicine, Cancer Cell, Cancer Research; Am J Pathology; Cell; Nature; Nature Reviews Immunology; Nature Reviews Cancer; Carcinogenesis
2006 Nature Reviews Cancer; Nature; Nature Medicine; Cell; Cancer Research; Clinical Cancer Research; J Exp Med; Cancer Cell: Am J Pathology; J Cell Biology
2007 Cell; Nature; PNAS: J Cell Biology; Cancer Research; J Exp Med; Breast Cancer Research
2008 Cancer Cell; PNAS; J Immunology; Nature;

VI. INVITED PRESENTATIONS

Symposia and Workshops: International

1996 *Human Tumor Heterogeneity II*: Cytometric Measurement of Growth Regulation and Genetic Alterations: International Society of Analytical Cytometry. Kananaskas, Alberta, Canada.
1997 *GeneMedicine-Boehringer Mannheim Cancer Alliance: Technology Workshop*. Cancún Mexico.
2001 *2nd Annual International Protease Society*. Freising, Germany.
2002 *6th International Symposium on Predictive Oncology & Intervention Strategies*, Pasteur Institute, Paris, France
2002 KEYNOTE LECTURE, *Dutch Cancer Society Annual Symposium*, Luntern, The Netherlands
2002 KEYNOTE LECTURE, *Cancer: Genome, Signal & Environment, Takeda Genome Urology International*, Kyoto, Japan
2003 *2nd Annual International Symposium on Epithelial Biology*, Timberline, Oregon USA
2004 10th International Congress of the *Metastasis Research Society*, 'Progress Against Tumor Progression', Genoa Italy
2005 *2005 International Consortium Meeting of the Children's Tumor Foundation: Molecular Biology of NF1, NF2 and Schwannomatosis,* Aspen, CO, USA
2005 *International Symposium on Systems Genome Medicine - Bench to Bedside,* Institute of Medical Sciences University of Tokyo, Tokyo, Japan
2005 *Immunotherapy of Cancer*, XI Annual Symposium of the Danish Cancer Society, Copenhagen, Denmark
2005 *4th General Meeting of the International Proteolysis Society*, Quebec City, Canada
2006 Centro Nacional de Investigaciones Oncológicas (CNIO) Cancer Conference: *Inflammation and Cancer*, Mardid SPAIN
2006 18th Annual Pezcoller Symposium *'Tumor Microenvironment: Heterotypic Interactions'*, Trento ITALY
2006 European Association for Cancer Research (EACR) 1st Annual Meeting, Budapest HUNGARY
2006 XXXIVth Meeting of the International Society for Oncodevelopmental Biology and Medicine (ISOBM: Tumor Biology, Detection and Therapy, Pasadena, CA, USA
2006 37th International Symposium of the Princess Takamatsu Cancer Research Fund '*Cancer Cells and Their Microenvironment*', Tokyo, JAPAN
2007 *4th International Conference on Tumor Microenvironment*, Florence, ITALY
2007 *2nd International Symposium on Cancer Metastasis and the Lymphovascular System*: Basis for Rational Therapy, San Francisco CA USA
2007 CNIO – Nature Symposium on "*Oncogenes and Human Cancer*". The Next 25 Years", Madrid SPAIN
2007 KEYNOTE LECTURE, *7th International Symposium on Hodgkin Lymphoma*, Cologne, GERMANY
2007 CANDLELIGHT LECTURE, *Inflammation and Cancer: From molecular links to bed side*; Inaugural meeting for the *Istituto Clinico Humanitas*, Milan ITALY

UPCOMING INVITATIONS

2008 *7th Annual International Congress on the Future of Breast Cancer*, Kauai, Hawaii USA
2008 Cancer Research UK Cambridge Research Institute (CRI) Inaugural Annual Symposium, '*Unanswered Questions in the Tumour Microenvironment'*, Homerton College, Cambridge UK
2008 5th Intl Kloster Seeon Meeting on Angiogenesis, Munich GERMANY
2008 NCRI Cancer Conference, Birmingham UNITED KINGDOM
2009 21ST Lorne Cancer Conference, Lorne AUSTRALIA
2009 19th Annual BioCity Symposium, 'Tumor Microenvironment in Cancer Progression", Tirku FINLAND

Symposia and Workshops: National

1994 *Current Transgenic Technology*, B & K Universal, San Mateo, CA, USA
1997 *Biology of Proteolysis*, Cold Spring Harbor Laboratory, NY, USA
1997 *Molecular Biology & Pathology of Neoplasia*, AACR, Keystone, CO, USA
1997 *Matrix Metalloproteinases*, Gordon Research Conference, Proctor Academy, New London, NH, USA
1998 *Proteolysis*, Gordon Research Conference, Colby-Sawyer College, New London, NH, USA
1998 *Cellular Targets of Viral Carcinogenesis*, AACR Special Conference. Dana Point, CA, USA
1998 *Mechanisms of Tumor Growth & Invasion Mediated by Proteolysis*, UCSF-Molecular Design Institute. San Francisco, CA, USA
1999 *Tumor Microenvironment*, Education Session, AACR Annual Meeting. Philadelphia, PA, USA
1999 *Matrix Metalloproteinases*, Gordon Research Conference, Colby-Sawyer New London, NH, USA.
2000 *Epithelial-Stromal Interactions & Tumor Progression Workshop*, National Cancer Inst., Bethesda, MD, USA
2000 10th National Conference of the Inflammation Research Association, Hot Springs, VA, USA
2001 *'Meet-the-Expert' Sunrise Session,* AACR Annual Meeting, New Orleans, LA, USA
2002 *Chemotherapy of Experimental & Clinical Cancer*, Gordon Research Conference, Colby Sawyer College, New London, NH, USA
2002 *Proteolytic Enzymes & their Inhibitors*, Gordon Research Conference, Colby Sawyer, New London, NH, USA
2002 *From the Cancer Cell to a Tumor - Tumors as Outlaw Organs,* Schilling Research Conference, The American Cancer Society, Aptos CA, USA
2002 *Cancer Intervention 2002*, Van Andel Research Institute, Grand Rapids, Michigan USA
2002 *Pathobiochemistry B Study Section Workshop,* Natl. Cancer Institute, Hilton Head, SC, USA
2002 *Proteases, Extracellular Matrix and Cancer*, AACR Special Conference, Hilton Head Island, SC, USA
2002 *ECM and Cancer*, Minisymposium, ASCB Annual Meeting, San Francisco, CA, USA
2003 *Matrix Metalloproteinases*, Gordon Research Conference, Big Sky, Montana, USA
2003 *Angiogenesis & Microcirculation*, Gordon Research Conference, Salve Regina, Newport R.I., USA
2003 *Inflammatory Cells and Cancer,* Symposium, American Society of Hematology 2003 Annual Meeting, San Diego, CA, USA
2003 *Validation of a Causal Relationship: Criteria to Establish Etiology*, National Cancer Institute, Cancer Etiology Branch, Washington, DC, USA.
2003 *Functional Imaging of Proteolysis*, Special Session, ASCB Annual Meeting, San Francisco, CA, USA
2004 Scleroderma Research Foundation Annual Scientific Workshop, San Francisco, CA, USA
2004 *Systems Biology of Cancer: The Tumor as an Organ,* Symposium, 95th AACR Annual Meeting. Orlando, FL, USA
2004 *Inflammation and Cancer,* Symposium, 95th AACR Annual Meeting. Orlando, FL, USA
2004 *Remarkable Role of the Microenvironment in Development and Disease Pathogenesis*, Symposium; Experimental Biology 2004, Sponsored by: the Assoc. of Anatomy, Cell Biology and Neurobiology, Washington, D.C., USA.
2004 *Molecular and Cellular Basis of Disease: Structure and Function of the Extracellular Matrix in Disease: Novel Roles and Regulation of MMPs and TIMPs in Disease,* Symposium; Experimental Biology 2004, Sponsored by: the Am. Society of Investigative Pathology, the American Society for Matrix Biology and the North American Vascular Biology organization. , Washington, D.C., USA.
2004 Pacific Coast Protease Workshop, Half Moon Bay, CA, USA.

2004 19th Aspen Cancer Conference: *Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention and Cancer Therapy.* Aspen, CO, USA.

2005 Keystone Symposia, *The Role of Microenvironment in Tumor Induction and Progression (J5),* Banff, Alberta CANADA

2005 Keystone Symposia, *Inflammation and Cancer (B8)*, Breckenridge, CO, USA

2005 *Symposium on Inflammation, Repair and Carcinogenesis in Liver, Pancreas and Colon*. UCSF Liver Center and the Program in Gastrointestinal Cancer of the UCSF Cancer Center, Rohnert Park, CA, USA

2005 *In the Forefront of Advances in Cancer Research*, Symposium, 96th AACR Annual Meeting. Anaheim, CA, USA

2005 *Macrophage Symposium*, AMGEN, Seattle, WA, USA

2005 *Immune Response to Cancer Symposium*, 41st Annual Meeting, American Society Clinical Oncology (ASCO), Orlando. FL. USA

2005 *Phagocyte*, Gordon Research Conference, New London, CT, USA

2005 *Mouse Models of Human Cancer Consortium*, Annual Steering Committee Meeting, New Brunswick, NJ USA

2005 *Matrix Metalloproteinases*, Gordon Research Conference, Big Sky, Montana, USA

2005 *Annual Buffalo Regional Conference on Immunology*, Buffalo, NY, USA

2005 2005 Montagna Symposium on *'Tissue repair - molecular mechanisms and clinical challenges'*, Salishan Lodge, OR, USA

2005 4th Annual AACR Conference on *Frontiers in Cancer Prevention Research*, Baltimore MD, USA

2005 AACR Special Conference, *Cancer, Proteases and the Microenvironment*, Bonita Springs, Florida. USA

2006 Timberline Annual Symposium on Epithelial Biology, *Intrinsic and Microenvironmental Regulation of Epithelial Cancer*', Timberline Lodge, Oregon, USA

2006 Keystone Symposium, *Molecular Targets for Cancer Prevention*, Granlibakken Resort, Tahoe City, CA, USA

2006 *Inflammation and Cancer,* Symposium, *97th AACR Annual Meeting*. Washington, D.C., USA

2006 Lineberger Cancer Center's *30th Annual Scientific Symposium*, University of North Carolina, Chapel Hill, North Carolina, USA

2006 KEYNOTE LECTURE, *Vanderbilt-Ingram Cancer Center Retreat 2006*, Vanderbilt University, Nashville TN, USA

2006 Tumor Biology Plenary Lecture, *Advances in Neuroblastoma Research 2006*, Los Angeles, CA, USA

2006 *Genetic, Cellular and Microenvironmental Determinants of Tumor Progression and Metastasis: A 'TPM' Workshop Honoring Martin L Padarathsingh, Ph.D.* TPM Study Section Workshop, Natl. Cancer Institute, Georgetown, VA, USA

2006 ASCO/Federation of European Societies Symposium: *Inflammation in Cancer Progression*, 2006 ASCO Annual Meeting, Atlanta, GA, USA

2006 AACR Special Conference, *Mouse Models of Cancer*, Cambridge, MA, USA

2006 AACR Special Conference, *Tumor Immunology: An Integrated Perspective.* Miami, FL, USA

2007 7th AACR-Japanese Cancer Association Joint Conference: *In the Forefront of Basic and Translational Cancer Research*, Waikoloa, Hawaii, USA

2007 Keystone Symposium, *'Mouse Models at the Frontiers of Cancer Discovery'*, Whistler, British Columbia, CANADA

2007 Keystone Symposium '*Inflammation and Cancer'*, Santa Fe, NM, USA

2007 AAAS Annual Meeting, *Healthy Aging: Inflammation and Chronic Diseases'* Symposium, San Francisco, CA USA

2007 Tumor Microenvironment and Tumor-Stromal Interactions Workshop: Sponsored by Biogen Idec Inc., Oncology Discovery Research, San Diego CA USA

2007 American Thoracic Society 2007 International Conference, *San Francisco Science: Inflammation, Immunity and Signaling*. San Francisco, CA USA

2007 22nd Aspen Cancer Conference: Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention and Cancer Therapy, Aspen CO, USA

2007 Gordon Research Conference, *Epithelial Differentiation & Keratinization*, Bryant University, Smithfield, RI, USA
2007 AACR, *Frontiers in Cancer Prevention Research Conference*, Philadelphia, PA, USA
2007 National Cancer Institute Workshop, '*Profiling of Immune Response to Guide Cancer Diagnosis, Prognosis and Prediction of Therapy'*, Bethesda, MD, USA
2008 47th Midwinter Conference of Immunologists, '*Meeting the challenge: Immunobiology in health and disease*', Asilomar, CA USA
2008 AACR-TREC-NCI Conference on *Energy Balance and Cancer: Mediators and Mechanisms*, Lansdowne, VA USA
2008 Keystone Joint Symposium, '*Cell Death in the Immune System / Cell Death and Cellular Senescence'*, Beaver Run Resort in Breckenridge, CO, USA
2008 Keystone Symposium, '*Inflammation, Microenvironment and Cancer'*, Snowbird Resort in Snowbird, Utah, USA
2008 The John F. Anderson Memorial Lectures in Medicine, '*The Linkage between Inflammation and Cancer*', University of Virginia, Charlottesville VA, USA
2008 *Tumor Microenvironment Symposium*, Stony Brook University, Stony Brook. NY. USA
2008 KEYNOTE LECTURE, Fox Chase Cancer Center 13th Annual Postdoctoral Fellow and Graduate Student Symposium, Philadelphia, PA USA

UPCOMING INVITATIONS
2008 DOD BCRP Era of Hope Meeting 2008, Symposium Session: *Immune and Inflammatory Contributions to Breast Cancer*, AND *Era of Hope Spotlight Session*, Baltimore MD, USA
2008 *AACR Centennial Conference: Translational Cancer Medicine 2008: Cancer Clinical Trials and Personalized Medicine;* Hyatt Regency Monterey in Monterey, CA USA
2008 *AACR Special Conference*, *Chemical and Biological Aspects of Inflammation and Cancer*, Ko Olina Hawai, USA
2008 International Society for Biological Therapy of Cancer (iSBTc), Workshop on Inflammation in Cancer Development, Westin Horton Plaza San Diego, CA USA
2008 *AACR Special Conference in Cancer Research, Tumor Immunology: New Perspectives*; Miami FL, USA
2008 Skirball Symposium, New York University School of Medicine, New York, NY USA
2009 Keystone Symposium, *Mobilizing Cellular Immunity for Cancer Therapy*, Snowbird Resort in Snowbird, UT USA
2009 Keystone Symposium, '*Extrinsic Control of Tumor Genesis*, Vancouver, British Columbia CANADA
2009 *1st Conference on Regulatory Myeloid Suppressor Cells*, Clearwater, FL USA
2009 2nd annual retreat of the CCR-NCI Cancer and Inflammation Program in Gettysburg, PA

Invited Lectures/Seminars: International
2000 Medical Genome Center, Division of Molecular Medicine, Australian National University, Canberra, A.C.T. Australia.
2001 German Cancer Center, Heidelberg, Germany.
2001 MERCK Pharmaceutical, Damstedt Germany.
2003 University of Toronto, Ontario Cancer Institute & Princess Margaret Hospital, Toronto, Ontario, CANADA
2004 Cancer Research UK, Barts & The London Queen Mary's School of Medicine & Dentistry, John Vane Science Center, Charterhouse Square, London, UK
2004 Cancer Research UK, London Research Institute, Lincoln's Inn Fields Laboratories, London, UK
2004 University of British Columbia, Department of Biochemistry and Molecular Biology, Vancouver, British Columbia, Canada
2007 Angiogenesis and Tumor Targeting Research Unit & Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, ITALY
2008 Institute of Cell Biology, ETH Zurich Switzerland

UPCOMING INVITATIONS

2009 McGill Cancer Center, McGill University, Montreal, Quebec, CANADA
2009 The Netherlands Cancer Institute, Amsterdam, THE NETHERLANDS

Invited Lectures/Seminars: National

1997 Biologic Therapy Research Conference. Univ. of Pittsburgh Medical Center, Pittsburgh, PA, USA
1997 Immunology Seminar Series. Univ. of Pittsburgh Medical Center, Pittsburgh, PA, USA
1999 Axys Pharmaceuticals, South San Francisco, CA, USA
1999 Berlex Pharmaceuticals, Emeryville, CA, USA
1999 Axys Pharmaceuticals, La Jolla, CA, USA
1999 14th Annual Excalibur Round Table, American Cancer Society, San Francisco, CA, USA
1999 Colloquium in Microbiology, Cell and Molecular Biology. San Francisco State Univ., San Francisco, CA, USA
2000 Chiron Corporation, Emeryville, CA, USA
2000 Oral and Pharyngeal Cancer Branch/NIDCR, National Institutes of Health, Bethesda, MD, USA
2000 Fibrogen, Inc., South San Francisco, CA, USA
2000 Scios Inc., Sunnyvale, CA, USA
2000 Molecular Biology Department, University of Southern California, Los Angeles, CA, USA
2001 Dept. of Pediatric Hematology and Oncology, Children's Hospital Los Angeles, Univ. of Southern California, Los Angeles, CA, USA
2001 Jonnson Comprehensive Cancer Center, Univ. of Calif., Los Angeles, Los Angeles, CA, USA
2002 Institute for Engineering and Medicine, Univ. of Pennsylvania, Philadelphia, PA, USA
2002 Oncology Grand Rounds, Univ. of Missouri, Columbia, MO.
2002 Cancer Center, Univ. of California, Davis, Davis CA, USA
2002 AstraZeneca, Waltham, MA USA
2002 Pharmacology Seminar Series, Dept. of Pharmacology, Wayne State Univ., Detroit, MI, USA
2003 Dept. of Biology, Univ. of Calif., San Diego, San Diego, CA USA
2003 Tularik, Inc., South San Francisco, CA USA
2003 Dept. of Cancer Biology's Cancer Metastasis Research Program Seminar Series, M.D. Anderson Cancer Center, Univ. of Texas, Houston, TX, USA
2003 Dept. of Cancer Biology, Stanford University, Stanford, CA, USA
2004 Burnham Cancer Institute, San Diego, CA, USA
2004 The Wistar Cancer Institute, Philadelphia, PA, USA
2004 Regeneron Pharmaceuticals, Inc. Tarrytown, New York, USA
2004 Keynote Lecture: Vanderbilt University Digestive Disease Research Center Retreat, Vanderbilt University, Nashville, TN, USA
2004 Dana Farber Cancer Center, Harvard Medical School, Boston MA, USA
2004 Indiana University, Herman B. Wells Center for Pediatric Research and Clinical Cancer Center, Indianapolis IN, USA
2004 Immunology Graduate Program Seminar, Stanford University, Stanford, CA, USA
2005 Dept. of Nutritional Sciences & Toxicology, Univ. of Calif., Berkeley, Berkeley, CA USA
2005 Rigel, Inc., South San Francisco, CA USA
2005 Dept of Pathology & Lab Medicine, Univ. of California, Los Angeles, Los Angeles, CA USA
2006 Division of Cancer Biology and Angiogenesis in the Department of Pathology at Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA USA
2006 Department of Molecular and Medical Pharmacology, University of California, Los Angeles, Los Angeles, CA USA
2007 *Lymphoma and Myeloma Conference*, M.D. Anderson Cancer Center, Houston, TX, USA
2007 University of Minnesota, Dept. of Lab Medicine and Pathology, Minneapolis, MN, USA
2007 Memorial-Sloan Kettering Cancer Center, Program in Cancer Biology and Aging, New York NY, USA
2007 Abramson Family Cancer Research Institute and Univ. of Pennsylvania, Division of Hematology-Oncology, Philadelphia, PA USA
2007 Albert Einstein College of Medicine, New York NY, USA

2007 Oncology Division Research, Biogen Idec Inc., San Diego, CA USA
2007 Genentech, Inc. Immunology Program. South San Francisco, CA USA
2007 University of Iowa Carver College of Medicine, Dept of Pathology, *Pathology Grand Rounds*, Iowa City, Iowa, USA
2007 Hollings Cancer Center, Medical University of South Carolina, Charleston, SC, USA
2007 University of Michigan, Program in Immunology and Cancer Research Series, Ann Arbor, MI USA
2008 Department of Pathology/UCLA School of Medicine Seminar, Los Angeles CA USA
2008 ANNUAL KEYNOTE LECTURE, Dept of Cancer Biology, Meharry Medical College, Nashville, TN USA
2008 University of California, Davis Cancer Center, Sacramento, CA USA
2008 Department of Immunology, University of Pittsburgh School of Medicine. Pittsburgh, PA, USA
2008 Cancer Biology Series, Ben May Cancer Center, University of Chicago, Chicago, IL, USA

UPCOMING INVITATIONS
2008 National Cancer Institute Center for Cancer Research Grand Rounds Series in Clinical and Molecular Oncology. Bethesda MD, USA
2009 Burnham Institute for Medical Research, San Diego CA, USA
2009 University of Michigan, Oral Health Sciences Program and Dental School Seminar Series, Ann Arbor, MI USA
2009 Molecular biology seminar series, Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Aurora, CO USA

Invited Lectures/Seminars: UCSF
1997 Breast Cancer SPORE Seminar. UCSF
1999 Cancer Research Institute Retreat, Tomales Bay, CA
2000 Chemistry and Cancer: How Chemistry-Based Tools Are Helping Solve Today's Serious Health Problems, Dev. & Alumni Relations, UCSF
2000 Oncology Grand Rounds, Department of Hematology and Oncology, UCSF
2000 PIBS-Cell Biology Seminar Series, UCSF
2000 Pathology and Lab Medicine Grand Rounds, UCSF
2000 BMS Student Pizza Talk, UCSF
2000 Cell Cycle & Dysregulation Club, Comprehensive Cancer Center, UCSF
2000 Comprehensive Cancer Center Retreat, Granlibakken, Tahoe City, CA
2001 BMS Student Pizza Talk, UCSF
2001 Pathology and Lab Medicine Grand Rounds, Departments of Medicine and Pathology, UCSF
2001 UCSF, Cell Biology Retreat, Wilbur Hot Springs, CA, USA
2001 UCSF TETRAD Retreat, Granlibakken, Lake Tahoe, CA, USA
2001 UCSF Cancer Research Institute/BMS Retreat, Granlibakken, Lake Tahoe, CA. USA
2002 Current Topics in Medical Science, UCSF Medical Scientist Training Program (M170.09)
2002 Mouse Models of Human Cancer Program, Comprehensive Cancer Center, UCSF
2002 Cancer Research Institute Retreat, Santa Cruz, CA
2003 PIBS Student Pizza Talk, UCSF
2003 Breast Oncology Program, Comprehensive Cancer Center, UCSF
2003 Comprehensive Cancer Center Faculty Retreat: *Identification and Functional Assessment of Cancer Effectors*, Golden Gate Club, San Francisco CA
2004 BMS Graduate Program Retreat, Granlibakken Tahoe City, CA
2005 BMS Student Pizza Talk, UCSF
2006 *Introduction to Research,* Department of Pathology, UCSF
2008 Division of Experimental Medicine, Divisional Seminar Series, UCSF

VII. GOVERNMENT AND OTHER PROFESSIONAL SERVICE:

GOVERNMENT SERVICE

2003-2006	National Institutes of Health, Center for Scientific Review	Ad hoc reviewer (10/2003; 02/2005; 10/2005; 06/2006), Tumor Progression & Metastasis (TPM) Study Section, Oncological Sciences Review group
2003	Division of Cancer Biology, National Cancer Institute: *Microenvironment Think Tank*	Participant and *Reporter*
2003	Division Cancer Etiology, National Cancer Institute: *Validation of A Causal Relationship: Criteria to Establish Etiology Think Tank*	Invited speaker and Participant
2004	National Institutes of Health, National Cancer Institute	Subcommittee C (05/2004) – Basic & Preclinical NCI Initial Review Group, NCI-C RPRB (T2) Angiogenesis
2005	National Institutes of Health, National Cancer Institute	Subcommittee D (02/2005) – Clinical Studies NCI Initial Review Group, NCI-D RPRB Tumor Pathology
2005	National Institutes of Health, Center for Scientific Review	Special Emphasis Panel (SEP); ZRG1 ONC (03) M, Developmental Therapeutics

OTHER PROFESSIONAL SERVICE

1999	Arkansas Science & Technology Authority	Ad hoc Grant Review
2000	McGraw-Hill, '*Biology*' 6th edition, Ed. P.H. Raven and G.B. Johnson	Ad hoc Review, Chapters 17 and 18
2000	Division of Cancer Biology, NCI: *Epithelial-Stromal Interactions & Tumor Progression Workshop*	Invited speaker and Participant
2001	Department of Veterans Affairs	Ad hoc Grant Review, Oncology Review Board
2001	Research Grants Council of Hong Kong	Ad hoc Grant Review
2003	Danish Cancer Society, DENMARK	Ad hoc Grant Review
2004	Division of Gastroenterology and Digestive Disease Research Center, Vanderbilt University, Nashville TN, USA	'*H. pylori-induced Inflammation and Gastric Adenocarcinoma*, PO1 External Advisory Panel
2004	Cancer Research Ireland, Irish Cancer Society	Ad hoc grant review
2004	Dutch Cancer Society	Ad hoc grant review
2004	Vanderbilt University, Nashville TN, USA; SPORE in GI Cancer	Ad hoc reviewer for SPORE Developmental Research Program
2005	Keystone Symposia, *Inflammation and Cancer*	Co-organizer (with Dr. Ray DuBois, Vanderbilt Univ, TN), Breckinridge, CO, USA
2006	5th Annual Timberline Symp. on Epithelial Cell Biology, '*Intrinsic and Microenvironmental Regulation of Epithelial Cancer*'	Co-Organizer (with Dr. Harold Moses, Vanderbilt University, TN, USA), Timberline, OR, USA
2006	Keystone Symposia Cancer Study Group for 2009 programming	Study group member
2007	Keystone Symposia, *Inflammation and Cancer*	Organizer (with Drs. Fran Balkwill (Cancer Research UK) and Glenn Dranoff (Beth Israel Cancer Center, Harvard, MA) Santa Fe, New Mexico, USA

2008	AACR Special Conference on '*Inflammation and Cancer*'	Co-Organizer (with Drs. Michael Karin and Larry Marnett)
2007-2010	External Scientific Advisory Board Member	University of Minnesota Cancer Center; Douglas Yee, M.D., Director
2007 – 2011	Member, External Scientific Advisory Board, CA U54 TMEN: *Significance of Microenvironment for Prostate Cancer Initiation and Progression*; P.I. Stephen R Plymate, Univ. of Washington School of Medicine.	University of Washington, Seattle WA, USA
2007 – 2011	Member, External Scientific Advisory Board, CA U54 TMEN: *Novel Methods for Detection Cell Interactions in the Tumor Microenvironment*; P.I. John S. Condeelis, Albert Einstein College of Medicine.	Albert Einstein College of Medicine of Yeshiva University, New York, NY USA
2008	International Society for Biological Therapy of Cancer (iSBTc), *2008 Workshop on Inflammation in Cancer Development*	Co-Organizer (with Drs. Michael Karin, Steven Dubinett, George Weiner)

VIII. UNIVERSITY AND PUBLIC SERVICE

UNIVERSITY SERVICE

System wide

1992-1993	Graduate Student Representative, Dept. of Biological Chemistry Faculty Council, UCLA
2004	*ad hoc* Member External Advisory Panel; Jonsson Comprehensive Cancer Center, University of California, Los Angeles, Los Angeles CA, USA

University of California, San Francisco (CAMPUS-WIDE)

1997	Presentation, Donor Seminar, UCSF Development Office
1998	Presentation, Donor Seminar, UCSF Development Office
2000 – 2004	Member, Steering Committee, Ovarian Cancer Program Project Grant
2000 – 2005	Member, Scholarships and Awards Committee, Academic Senate, School of Medicine
2002 – present	Member, BioMedical Sciences Graduate Program (BMS) Executive Committee
2002 – 2004	Member, Medical Scientist Training Program Executive Committee
2004 - 2006	Member, Search Committee, Director of Molecular Imaging, Dept. of Radiology, Committee Chair: Ron Arenson, M.D. no successful recruitment
2004 - present	Member, BioMedical Sciences Graduate Program (BMS); Admissions Committee
2004	Organizer, BioMedical Sciences Graduate Program Retreat, Granlibakken, N. Lake Tahoe, CA USA
2005 - 2006	Member, Tissue Engineering Ladder-rank Faculty Search Committee, Dept. of Surgery, Committee Chair: Nancy Boudreau, Ph.D. Successful recruitment of Valerie Weaver, Ph.D.
2005 - present	Member, Ethel and Jane Sokolow Memorial Cancer Endowment Lectureship Committee.
2006	Member, Cancer Faculty Search Committee, Anatomy Dept., Committee Chair: Zena Werb, Ph.D. Successful recruitment of Jeroen Roose, Ph.D.
2006	Member, Faculty Advisory Committee for 2007 Journalist Seminar on *Inflammation and Disease*. Sponsored by Associate Vice Chancellor Barbara J. French
2007	Member, committee to select recipient of Dean's Postdoctoral Prize Lecture.
2007	Member, Faculty Search Committee for Restorative Neurosurgery and Stem Cell Neurobiology, VA Medical Center/UCSF NeuroSurgery. Committee Chair: Linda Noble, Ph.D.; Status: open.

University of California, San Francisco, Comprehensive Cancer Center

1999 Member, Cancer Center Research Building Space Review Policy Committee
1999 – 2002 Member, Mt Zion Animal Barrier Facility Committee
1999 – 2005 Member, Cancer Center Friday Seminar Series Committee
2000 Organizer and Chair, MZ Cancer Center Research Building Annual Retreat
2001 Member, 'Star Performance Award' selection committee
2001 Presentation, Evelyn Herman Reception, UCSF Development Office
2001 – 2002 Member, Cancer Center Research Building, 'Cancer Center Faculty Working Group'
2001 – 2006 Member, Mouse Models of Human Cancer Working Group
2002 – 2003 Member, UCSF Mt Zion campus, Animal Protocol Review Committee
2002 Member, ACS IRG grant review committee
2002 – 2006 Steering Committee Member, Mouse Models of Human Cancer
2003 Member, Review Committee, UCSF Comprehensive Cancer Center Stewart Trust Award
2003 – present Chair, UCSF Mt Zion Campus Animal Protocol Review Committee
2003 Member, Search Committee: Associate Director for Administration, UCSF Comprehensive Cancer Center (Erica Weber, recruited)
2004 Member, Review Committee, UCSF Comprehensive Cancer Center Stewart Trust Award
2006 Organizer, UCSF CCC Annual Symposium, *'Inflammation & Cancer: Bench to Bedside'.*
2008 Chair, Committee to nominate Postdoctoral scholar for AACR 2008 Annual Meeting, Inaugural "Future Leaders, New Directions" Special Symposium. Nominee: Laura Soucek, Ph.D. (awarded)

University of California, San Francisco, Cancer Research Institute

2001 – 2002 Member, Cancer Research Institute Membership Subcommittee

University of California, San Francisco, Department of Pathology

2003 Member, Committee to recommend faculty for the *Robert E. Smith Endowed Chair in Experimental Pathology*
2004 Member, Search Committee, Ladder rank faculty, Physician-Scientist, Anatomic Pathology. Successful recruitment of Jay Debnath, M.D., Ph.D.
2007 Member, Search Committee, Ladder-rank faculty, Physician-Scientist, Pathology and Neuropathology. Committee Chair: Michael D Prados, M.D.; Status: open.
2008 Member, Search Committee, Ladder-rank faculty, Physician-Scientist, Experimental Pathology. Committee Chair: Benedict Yen, M.D.; Status: o[en

University (other)

2002 Guest Instructor, Graduate Oncology, University of Missouri, Columbia, Missouri USA
2003 Guest Instructor, Cancer Biology, Stanford University, Stanford, CA USA
2004 Guest Instructor, Immunology, Stanford University, Stanford, CA USA
2008 Guest Instructor, *Exploring the Tumor microenvironment*, Postgraduate course, ISREC, Lausanne University's Biochemistry and Biology Departments, and the Lausanne Branch of the Ludwig Institute, Lausanne Switzerland. Course Organizers, Ivan Stamenkovic and Michel Aguet

PUBLIC SERVICE:

1990 Lecturer, Science Academy Of Whittier, Summer Institute. Whittier College, Whittier, CA
1991 Organizer and Lecturer, Science Academy Of Whittier, Summer Institute. Whittier College, Whittier, CA.
1993 Lecturer, Joslyn Community Center. Claremont, CA.
1994 Provided elementary educators with science-related supplies (photos, slides, fixed tissue samples).
1995 Co-Coordinator Hormone Research Institute, 'Take Our Daughters To Work Day', Univ. of Calif., San Francisco

2002 Photo credits and interviewed for '*Misdiagnosis: Failure of Promising Cancer Treatment Starts Soul Searching by Researchers & Drug Companies',* in: *San Francisco Chronicle,* May 12, 2002.

2003 Interviewed for article '*Body's First Defense May Be Root of Diseases',* in: *The Washington Post,* February 20, 2003

2003 Interviewed for article '*The Body on Fire*', in: U.S. News & World Report, October 20, 2003

2004 Interviewed for comments in: *Science News*, '*Early Warming: Inflammatory protein tied to colon cancer risk*" February 7, 2004, Vol 165.

2004 Interviewed for article '*The Fires Within'*, in: *TIME* Magazine, February 23, 2004

2004 Interviewed for comments on AACR Annual Meeting in: *Oncology Times*, '*Exercise Reduces Inflammatory Response, May also Reduce Cancer Risk*', Robert H Carlson, 26(11):33-34, June10, 2004

2004 Interviewed for article *'Inflammation and Cancer: The Link Grows Stronger',* in: *Science*, 306, 966-968 (2004)

2005 Interviewed for article *'Quieting a Body's Defenses',* in: *Newsweek*, Special Edition, Summer 2005

2006 Interviewed for "Expert Commentary" by *BreastLink.org,* on article "Association Between Circulating White Blood Cell Count and Cancer Mortality." *Archives of Internal Medicine*, January 23, 2006; 166:188-194. http://www.breastlink.org/index.php?module=announce&ANN_user_op=view&ANN_id=208

2007 UCSF Research Perspectives 2007 – Inflammation as Cause and Consequences of Disease, Media Event for Journalists, September 27, 2007, UCSF Mission Bay Campus

2007 On-Air radio interview by Dave Iversen, KQED *FORUM*, September 28, 2007 San Francisco CA USA

IX. TEACHING AND MENTORING

Formal Scheduled Classes for UCSF Students:

Qtr	Academic Yr	Course No. & Title	Teaching Contribution	Units	Class Size
W	1997/98	IDS 100; Histology Laboratory	*Neoplastic Skin Histopathology*; Laboratory lecture & instruction	10	150
W	1998/99	IDS 100; Histology Laboratory	*Neoplastic Skin Histopathology;* Laboratory lecture & instruction	10	150
W	1999/00	IDS 100; Histology Laboratory	*Neoplastic Skin Histopathology*; Laboratory lecture & instruction	10	150
S	1999/00	BMS 297A; Molecular Biology & Pathology of Neoplasia	*Animal Models of Cancer Laboratory*; Laboratory lecture & instruction	3	15
S	2000/01	BMS 297A; Molecular Biology & Pathology of Neoplasia	*Animal Models of Cancer Laboratory*; Laboratory lecture & instruction	3	15
W	2000/01	BMS 225; Tissue and Organ Biology	Lecture and laboratory instruction	3	15
S	2000/01	BMS 260; Cell Biology	Discussion group leader	1	6
F/W	2001/02	IDS 101; Prologue	Laboratory Instructor	9	30
W	2001/02	BMS 225; Tissue and Organ Biology	Lecture and laboratory instruction	3	15
W	2001/02	IDS 103; Cancer Block	*Invasion & Metastasis*; Lecturer	7	150
S	2001/02	BMS 260; Cell Biology	Discussion group leader	1	7
F	2002/03	BMS 260; Cell Biology	Discussion group leader	1	6

W	2002/03	IDS 103; Cancer Block	*Invasion & Metastasis*; Lecturer	7	150
F/W	2002/03	IDS 101; Prologue	Laboratory Instructor	9	30
F	2003/04	BMS 260; Cell Biology	Discussion group leader	1	6
S	2003/04	BMS 225B, Tissue and Organ Biology	Lecturer and Laboratory Instructor	1.5 - 5	tbd
W	2003/04	Biochem 297; Molecular Biology & Pathology of Neoplasia	*Angiogenesis:* Lecturer	3	30
W	2003/04	BMS 297A Molecular Biology & Pathology of Neoplasia Laboratory	Lecturer and Laboratory Instructor, *Animal Models of Neoplasia*	1	10
S	2003/04	BMS 225B; Tissue & Organ Biology	Lecturer: Cancer I & Cancer II	1.5 - 5	16
F	2004/05	BMS 260; Cell Biology	Discussion group leader	1	6
F	2005/06	BMS 260; Cell Biology	Discussion group leader	1	7
W	2006/07	Biochem 297; Molecular Biology & Pathology of Neoplasia	*Inflammation and Cancer*: Lecturer	3	30

Postgraduate and Other Courses:

1989	M204, *Biochemistry Lab* Univ. of Calif., Los Angeles	Student Teaching Assistant for quarter long course (100 medical students)
1989	Biology 250, *Human Heredity*; Dept. of Biology Whittier College, Whittier CA	Organized and taught entire lecture-based course (30 undergraduate students)
1990	Biology 350 & 350L, *Molecular Genetics*; Dept. of Biology, Whittier College, Whittier CA	Organized and taught entire lecture and laboratory course (16 undergraduate students)
1990	M204, *Biochemistry Lab* Univ. of Calif., Los Angeles	Student Teaching Assistant for quarter long course (100 medical students)
1990	Biology 250, *Human Heredity*; Dept. of Biology Whittier College, Whittier CA	Organized and taught entire lecture-based course (30 undergraduate students)
1992	Biology 350 & 350L, *Molecular Genetics*; Dept. of Biology, Whittier College, Whittier CA	Organized and taught entire lecture and laboratory course (16 undergraduate students)
2003	Graduate *Oncology*, University of Missouri, Columbia, MS, USA	Invited Guest Lecturer: Lecture syllabus & delivered 2-hr lecture for course (15 students, combination of graduate, medical & postgraduate fellows
2003	Graduate Program in Cancer Biology, Stanford Univ., Stanford, CA USA	Invited Guest Lecturer: Delivered 1-hr lecture to graduate students in Cancer Biology Graduate program
2004	Graduate Program in Immunology, Stanford Univ., Stanford, CA USA	Invited Guest Lecturer: Delivered 1-hr lecture to graduate students in Immunology Graduate program
2005	UCSF Dermatology residents' Basic Science Seminar Series	Invited Guest Lecturer: Delivered 1-hr lecture to UCSF Dermatology Residents (11 M.D. and M.D., Ph.D. Residents)
2008	ISREC, Lausanne University's Biochemistry and Biology Departments, and the Lausanne Branch of the Ludwig Institute	Guest Instructor: *Exploring the Tumor microenvironment*, postgraduate course. (20 PhD students, 3 hours of instruction)

High School and Undergraduate Students Supervised or Mentored:

Dates	Name	Program or School	Faculty Role	Current position
1998	Christopher Tinkle	Undergraduate, Univ. of Texas, Austin, TX, USA	Summer Research Training Program Supervisor	MSTP student, Rockefeller University
2000	Adam Zucker	Undergraduate, Oberlin College, Ohio USA	Supervised Summer work	unknown
2000	Ashkan Hirari	Undergraduate, Univ. of Calif., Berkeley, Berkeley CA, USA	Supervised Summer work	unknown
2001	Jason Reuter	Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA	Supervised Summer work	Ph.D. student, Stanford University
2002	Destinee Cooper	Undergraduate, Univ. of Calif., Davis USA	Summer Research Training Supervisor	unknown
2006	Sunum Mobin	UCSF Science & Health Education Partnership: High School Intern Program	Summer Research Training Supervisor	Intern, Coussens lab

Predoctoral Students Supervised or Mentored:

Dates	Name	Program or School	Faculty Role	Current position
2000	Jin-Sae Rhee	UCSF MSTP/BMS, graduate student	Rotation Supervisor	PhD awarded 2003, M.D. awarded 2005
2000 - 2003	Jin-Sae Rhee	UCSF M.D., Ph.D.,	Ph.D. supervisor	Pediatric Resident, Children's Hospital, Washington D.C.
2000	Maria Christophorou	UCSF BMS, graduate student	Faculty coach, BMS 297	Ph.D. awarded 2006
2001	Leslie Chu	UCSF BMS, graduate student	Rotation Supervisor	Ph.D. awarded 2005
2001	Rayna Takaki	UCSF BMS, graduate student	Rotation Supervisor	Ph.D. awarded 2006
2001 – 2002	Sophia Bruggerman	University of Nijmegan, The Netherlands	Masters Thesis Supervisor	Ph.D. student, The Netherlands Cancer Institute
2002	Lucy Lebedeva	UCSF PIBS, graduate student	Faculty coach, BMS 297	Ph.D. awarded 20057
2002	Leslie Chu	UCSF BMS, graduate student	Ph.D. Orals committee	Ph.D. awarded 2005
2002	Andre Whitkin	MSTP student, Cornell University USA	Supervised Summer work	MSTP student, Cornell University
2002	Karin deVisser	The Netherlands Cancer Institute, The Netherlands	Ph.D. Thesis Reading Committee	Postdoctoral fellow, The Netherlands Cancer Institute
2003	Cathy Collins	UCSF MSTP student	MSTP Advisor	MSTP student, UCSF
2004	Eric Tamm	University of British Columbia, Canada	Doctoral Dissertation External Examiner	Postdoctoral fellow, Genentech Inc.,
2004	Annie Hsieh	University of	Masters Thesis	unknown

		Södertörn, Sweden	Supervisor	
2005	Geoff Benton	UCSF TETRAD/PIBS, graduate student	Ph.D. Orals committee	UCSF TETRAD PhD graduate student
2006	Morgan Truitt	UCSF BMS, graduate student	Rotation Supervisor	UCSF BMS PhD graduate student
2006	Danielle Shin	UCSF MSTP student	Rotation Supervisor	MSTP student, UCSF
2006-present	Celeste Rivera	SFSU/UCSF NIH Post-baccalaureate Research Experience Program (PREP) student	M.S. research advisor	current MS student, Coussens lab UCSF
2007-present	Leslie Vasquez	SFSU/UCSF NIH Post-baccalaureate Research Experience Program (PREP) student	M.S. research advisor	current MS student, Coussens lab UCSF
2008	Ashley Martin	UCSF BMS, graduate student	Rotation Supervisor	UCSF BMS PhD graduate student

Postdoctoral Fellows and Residents Directly Supervised or Mentored

Dates	Name	Position & Funding	Faculty Role	Current Position
2000 - 2001	Ernst Lengyel, M.D., Ph.D.	Post-Doc Researcher, Senior Clinical Fellow	Research Supervisor	Assoc. Adj. Prof., Dept. Gyn. & Oncology, UCSF
2000 -2002	Leon Van Kempen, Ph.D.	Post-Doc Researcher, Dutch Cancer Society Postdoctoral Fellowship	Research Supervisor	Asst Prof., Univ. of Nijmegen, Dept. of Pathology, The Netherlands
2002 – 2005	Robert Diaz, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Scientist, Roche Pharmaceuticals
2002 – 2005	Karin deVisser, Ph.D.	Post-Doc Researcher, Dutch Cancer Society Postdoctoral Fellowship	Research Supervisor	Research Scientist, The Netherlands Cancer Institute, Amsterdam, The Netherlands
2003 – 2007	Alexandra Eichten, Ph.D.	Post-Doc Researcher, Serono Foundation for the Advancement of Medical Science (2003-2005); Coussens R01	Research Supervisor	Scientist, Regeneron Corp., New York USA
2003 - 2005	Stephen Robinson, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Private sector, United Kingdom
2003 - 2004	H. Jennifer Shen, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Post-Doctoral fellow, NIH
2005 - present	David DeNardo, Ph.D.	Post-Doc Researcher; 1) NGA: 5 T32 CA09043 PI: BISHOP; *The Molecular Analysis of Tumor Viruses;* 2) American Cancer	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF

		Society Postdoctoral Fellowship 2007-2010		
2005 – 2007	Nor Eddine Sounni, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Post-Doctoral fellow, Strongin Lab, The Burnhm Inst., San Diego CA USA
2006 – 2007	Tingting Tan, M.D.,Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Resident, Internal Medicine, Kaiser San Francisco
2006 - present	Magnus Johansson, Ph.D.	Post-Doc Researcher; Sweedish Cancer Society Postdoctoral fellowship 2006-2008	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF
2006- present	Nessrine Affara, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF
2007 - present	Pauline Andreu, Ph.D.	Post-Doc Researcher; Cancer Research Institute Postdoctoral Fellowship 2008-2011	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF

FACULTY MENTORING

Faculty Mentored:

Dates	Name	Position while Mentored	Mentoring Role	Current Position
2001 – 2004	Ernst Lengyel, M.D., Ph.D.	Asst. Adjunct Professor	Research Mentor	Asoc. Prof., Dept. Gyn. & Oncology, Univ. of Chicago, Chicago, IL
2002 – 2007	Darya Soto, M.D.	Asst. Adjunct Professor,	K08 Research Mentor	Private Practice, Burlingame, CA
2005 – 2007	Runi Chattopadhyay, M.D.	Clinical Instructor and Clinical Fellow	Basic Science Mentor, K12	Director, Breast Center, California Pacific Med. Center, San Francisco CA
2006 – present	Limin Liu, Ph.D.	Assistant Professor	Member, Mentoring Committee	Dept. of Microbiology & Immunology, Sandler Center for Basic Research in Asthma, UCSF

Sabbatical Visitors:

1999 - 2000 Yves DeClerck, M.D. Professor, Univ. of Southern Calif. & Children's Hospital of Los Angeles

SUMMARY OF TEACHING HOURS

Academic Year	Teaching/Mentoring Summary	Hours
1997/98	Total hours of teaching /mentoring:	27

	Formal class or course teaching hours: Informal teaching hours including prep time: Mentoring hours:	2 1 24
1998/99	Total hours of teaching /mentoring: Formal class or course teaching hours: Informal teaching hours including prep time: Mentoring hours:	71 2 1 68
1999/00	Total hours of teaching /mentoring: Formal class or course teaching hours: Informal teaching hours including prep time: Mentoring hours:	108 4 2 102
2000/01	Total hours of teaching /mentoring: Formal class or course teaching hours: Informal teaching hours including prep time: Mentoring hours:	130 16 9 105
2001/02	Total hours of teaching /mentoring: Formal class or course teaching hours: Informal teaching hours including prep time: Mentoring hours:	201 18 19 164
2002/03	Total hours of teaching /mentoring: Formal class or course teaching hours: Informal teaching hours including prep time: Mentoring hours:	314.5 15.5 17 282
2003/04	Total hours of teaching /mentoring: Formal class or course teaching hours: Informal teaching hours including prep time: Mentoring hours:	402 20 28 354
2004/05	Total hours of teaching /mentoring: Formal class or course teaching hours: Informal teaching hours including prep time: Mentoring hours:	395 17 28 350
2005/06	Total hours of teaching /mentoring: Formal class or course teaching hours: Informal teaching hours including prep time: Mentoring hours:	395 17 28 350
2006/2007	Total hours of teaching /mentoring: Formal class or course teaching hours: Informal teaching hours including prep time: Mentoring hours	473 45 28 400

X. RESEARCH AND CREATIVE ACTIVITIES

RESEARCH AWARDS AND GRANTS:

CURRENT

P01 CA72006 (PI: Werb, Z; UCSF) 07/07/03 – 06/30/08
Source: NIH/NCI $1,523,691 directs/yr 6
Title: *Proteases in Cancer Biology and Drug Development* $6,354,685 directs/yr 6-11
Project 3 - *Proteases in Models of Tumor Initiation/Progression* $229,788 directs/yr 6
Role: Co-Investigator, Project 3 $1,172,879 directs/yr 6-11
The major goal of this project is to study the role of proteases in cancer biology.
Core C - *Transgenic Animal Models* $151,612 directs/yr 6
Role: Director $765,974 directs/yr 6-11

The major goal of this Core is to develop and provide protease null and transgenic mice to program projects.

R01 CA98075 (PI: Coussens, LM; UCSF) 07/1/03-06/30/09 (1-yr no cost extension)
Source: NIH/NCI $222,500 directs/yr 1
Title: *Microenvironmental Regulation of Tumor Progression* $1,112,500 directs/yr 1-5
Role: Principal Investigator
The overall goal of this grant is to determine the role of collagen metabolism on epithelial carcinogenesis.

P30 CA82103 (PI: McCormick, F; UCSF) 8/5/1999-5/31/2012
Source: NIH/NCI $5,058,311 (directs)
Title: *Cancer Center Support Grant*
Role: Co-Director, Mouse Pathology Shared Resource
The Cancer Center Support Grant provides support for administration and infrastructure for the UCSF Comprehensive Cancer Center. Dr. Coussens is the Core Co-Director of the Mouse Pathology Shared Resource that provides routine hematologic and histopathologic processing of tissue and blood samples to members of the UCSF community.

BC051640 Era of Hope Scholar Award (PI: Coussens, LM; UCSF) 06/01/06 – 05/31/11
Source: Department of Defense Breast Cancer Research Program (BCRP) of tl $454,111 directs yr 1
of the Congressionally Directed Medical Research Programs (CDMRP**)** $2,347,728 total directs
Title: Microenvironment Regulation of Mammary Carcinogenesis
Role: PI
The goal of this Scholar Award is to identify leukocytes and their proteases that modify breast carcinogenesis and to develop noninvasive imaging reagents targeting leukocytes to image inflammation.

RO1 CA132566 (multi PI: Coussens, LM; Jablons DM; UCSF) 05/01/08-04/30/13
Source: NIH/NCI $190,000 directs/yr
Title: Inflammation and Lung Carcinogenesis $1,467,591 directs/yr 1-5
Role: Principal Investigator
The goal of this study is to determine how inflammation and Wnt signaling regulate stem cell niche autonomy during lung carcinogenesis

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RO1 CA130980-01A1 (PI: Coussens, LM) Proposed: 07/01/08-06/30/13
Source: NIH/NCI Proposed: $225,000 directs/yr
Title: Regulation of Inflammation-Associated Epithelial Cancer Development Priority Score: 148
Percentile: 12.5
Pending Council Review
Role: Principal Investigator
The goal of this study is to determine regulatory programs activating chronic inflammation during squamous carcinogenesis

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PENDING

CDMP/DOD (multiPI: Coussens, LM [UCSF], Love, S [DSLRF]) Proposed: 01/01/09-12/31/10
SRG review date: 09/08
Source: DOD, Synergistic Idea Award Proposed: $250,000 directs/yr
Title: Exploring the link between inflammation and risk-Examining macrophages in ductal fluid as an innovative indicator of the predisposition to breast cancer'
Role: Principal Investigator
The goal of this study is to investigate whether nipple aspirate fluid represents a low grade chronic inflammatory state that fosters future development of cancer

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PREVIOUS

USPHS 5 T32 CA09056 (PI: Fox, F, UCLA) 07/01/89 –06/30/92
Source: NIH/UCLA $9,300 directs/yr1
Title: Regulation of *junB* Gene Expression by TGF-Beta $25,800 directs/yr 1-3
Competitive Pre-Doctoral award to study transcription factor *junB.*

Univ. of Calif., Dissertation Year Fellowship (PI: Coussens, LM, UCLA) 10/1/92 – 09/31/93
Source: University of California, Office of the President $13,350 directs/yr
Title: *Effects of E1A on TGF-Beta-inducible junB Expression*
Competitive Pre-Doctoral award to study transcription factor *junB.*

USPHS 5 T32 CA09043 (PI: Bishop, KM, UCSF) 10/01/93-06/31/96
Source: NIH/UCSF $25,000 directs/yr
Title: *Molecular Analysis of Tumor Viruses* $75,000 directs/yr 1-3
Post-Doctoral fellowship to study mouse model of epithelial carcinogenesis.

American Social Health Association/Pfizer Post-Doctoral Research Fellowship in Sexually Transmitted Diseases (PI: Coussens, LM, UCSF) 10/01/96 – 9/30/98
Source: Private Foundation $27,500 directs/ yr 1
Title: *Metalloproteinases and Malignant Progression of Squamous Epithelium in K14-HPV16 Transgenic Mice* $56,250 directs/yr 1-2
Role: Principal Investigator
Competitive Post-Doctoral fellowship to study proteases and tumor development .

P01 CA072006 (PI: Shuman M, UCSF) 06/10/97 – 06/30/03
Source: NIH/NCI $803,021 directs/yr 1
Title: *Proteases in Cancer Biology and Drug Development* $4,280,649 directs/yr 1-5
Project 3 – Proteases in Models of Tumor Initiation/Progression $165,438 directs/yr 1
Role: Co-Investigator, Project 3 $940,281 directs/yr 1-5
The major goal of this project is to study the role of proteases in cancer biology. $72,595 directs/yr 1
Core C – Transgenic Animal Models $470,620 directs/yr 1-5
Role: Director (year 4 and 5)
The major goal of this Core is to develop and provide protease null and transgenic mice to program projects.

UCSF IRG-97-150-01 (PI: Coussens LM, UCSF) 07/01/99-06/30/00
Source: American Cancer Society $20,000 directs/yr 1
Title*: Proteases and Genomics in a Mouse Model of Epithelial Cancer* $20,000 directs/yr 1
Role: Principal Investigator
Pilot project tested role of proteinases as effectors of genomic instability.

UCSF Cell Cycle and Dysregulation Program (PI: Coussens LM, UCSF) 02/01/00-01/31/01
Source: UCSF Comprehensive Cancer Center, Intramural $14,000 directs/yr 1
Title*: Epithelial Neoplastic Progression and Degradation of Type ICollagen* $14,000 directs/yr 1
Role: Principal Investigator
Pilot project assessed functional significance of type I collagen metabolism during epithelial carcinogenesis.

Research Evaluation and Allocation Committee (PI: Coussens LM, UCSF) 07/01/00-06/30/01
Source: UCSF Academic Senate $30,000 directs/yr 1
Title: *Role of Gelatinase B in Maintenance of Genomic Instability* $30,000 directs/yr 1
Role: Principal Investigator
Pilot project tested the role of MMP9 as an indirect regulator of genomic instability.

UCSF IRG AC-04-02 (PI: Coussens LM, UCSF) 10/1/00-09/30/01
Source: American Cancer Society $20,000 directs/yr 1
Title: *Regulation of Intracellular Signaling Pathways by Gelatinase B/MMP-9* $20,000 directs/yr 1
Role: Principal Investigator
Pilot project to study signal transduction pathways regulated by MMP-9.

The V Foundation for Cancer Research (PI: Coussens LM, UCSF) 06/02/00-05/31/02
Source: Private Foundation $50,000 directs/yr 1
Title*: Gelatinase B and Epithelial Cancer Development* $100,000 directs/yrs 1-2
Role: Principal Investigator
Pilot project to study role of MMP9 during epithelial carcinogenesis.

Gertrude B. Elion Cancer Research Award (PI: Coussens LM, UCSF) 07/1/01 – 06/30/02
Source: American Association of Cancer Research $50,000 directs/yr 1
Title: *Functional Role of MMP-2 During Epithelial Carcinogenesis* $50,000 directs/yr 1
Role: Principal Investigator
Pilot project to study role of MMP-2 during epithelial carcinogenesis.

Univ. of Calif., Cancer Research Coordinating Committee (PI: Coussens LM, UCSF) 07/1/01 – 06/30/02
Source: University of California $48,874 directs/yr 1
Title: *Gelatinase A/MMP-2 and Epithelial Cancer Development* $48,874 directs/ yr 1
Role: Principal Investigator
Pilot project to study role of MMP-2 as a potentiator of tumor development.

Hellman Family Award For Early Career Faculty (PI: Coussens LM, UCSF) 11/1/00-09/30/02
Source: UCSF Intramural $49,000 directs/ yr 1
Title: *Paracrine Regulation of Epithelial Carcinogenesis by MMP-9* $89,000 directs/yr 1-2
Role: Principal Investigator
Pilot project to identify matrix molecules regulated by MMP-9.

Edward Mallinckrodt, Jr. Foundation (PI: Coussens LM, UCSF) 10/1/00-09/30/03
Source: Private Foundation $61,000 directs/yr 1
Title: *Regulation of epithelial cancer by gelatinase B/MMP-9* $194,000 directs/yr 1-3
Role: Principal Investigator
Pilot project to determine how MMP-9 regulates proliferation, VEGF bioavailability and angiogenesis during epithelial carcinogenesis.

P50 CA58207 (PI: Gray, J: UCSF) 03/01/03-02/28/05
Source: NIH/NCI $50,000 directs/yr 1
Bay Area Breast Cancer Translational Research Program (SPORE) $100,000 directs/yr 1-2
Title: *Type I Collagen Remodeling and Mammary Carcinogenesis*
Role: Principal Investigator (Developmental Project)
The overall goal of this pilot project was to explore the role of collagen metabolism during mammary carcinogenesis.

DE-FG02-05ER6401 (PI: Franc, B**;** UCSF**)** 03/01/05 – 01/16/06

Source: DOE Medical Applications Grant $225,100 directs yr 1
Title: Therapeutic Radionuclide Tumor-targeting Strategy for Breast Cancer $1,125,500 total directs
Role: Co-Investigator
The specific aim of this project is to develop a radionuclide delivery molecule (RDM) that specifically targets cancer cells that express matrix-metalloproteinase-14 (MMP-14) on their surface and demonstrate delivery of radiolabeled RDM to MMP-14 expressing cells *in vitro* and *in vivo.*

R01 DK067678 (PI: Cher, M: Wayne State University) 7/1/03-6/30/06
Source: NIH/NIDDK $14,675 directs/yr 1
Title: *Proteases in Prostate Cancer Bone Metastasis* $122,794 directs/yr 1-4
Role: Subcontract Principal Investigator
The major goal of this subcontract is to assist with the planned experiments by providing mice (protease deficient) of defined genotype for proposed studies to analyze proteases during prostate metastasis to bone *in vivo*.

Opportunity Award, Sandler Family (PI: Coussens, LM; UCSF) 02/15/05 -02/14/07
Source: UCSF Intramural $95,000 directs yr 1
Title: *B Lymphocytes as Targets for Cancer Prevention* $191,000 total directs
Role: Principal Investigator
The major goal of this project is to investigate the efficacy of targeting B cells for chemoprevention

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DAMD17-02-1-0693 (PI: Sloane, B; Wayne State University) 08/01/02-07/31/06
Source: Department of Defense $5,746,832 directs/yr 1-4
Breast Cancer Center of Excellence $49,576 directs/yr 1
Title: *Validation of Proteases as Therapeutic Targets in Breast Cancer Functional Imaging of Protease Expression, Activity and Inhibition*
Role: Subcontract Principal Investigator $198,307 directs/yr 1-4
The goal of this program is to validate proteases as therapeutic targets in breast cancer by functional imaging of protease expression, activity and inhibition.

R01 CA94168 (PI: Coussens, LM: UCSF) 04/1/02-06/31/07
Source: NIH/NCI $222,500 directs/yr 1
Title: *Regulation of Epithelial Cancer by MMP-9/gelatinase B* $1,112,500 directs/yr 1-5
Role: Principal Investigator
The goal of this project is to identify molecules that mediate proliferative and cellular pathways activated by MMP-9.

U54 RR020843 (PI: Smith, J; Burnham Institute) 09/30/04-07/31/06
Source: NIH/National Center for Research Resources $1,916,878 directs/yr 1-5
Title: Center on Proteolytic Pathways
Role: Principal Investigator (Driving Biological Problem #1) $67,306 directs/yr
DBP#1 *Proteolytic Pathways in Acute Vascular Response*

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2. Egeblad M, Wiseman BS, Sternlicht MD, Green KA, Kouros-Mehr H, DeNardo D, Wilcox J, Bissel MJ, **Coussens LM**, Lunf LR, Werb Z. Matrix metalloproteinase-dependent remodeling of the collagen scaffold regulates mammary epithelial invasion. *Manuscript submitted*
3. Gerg M, Kopitz C, Bäumler P, Pennington C, St-Pierre Y, Edwards D, **Coussens LM**, Krüger A. Identification of a pro-metastatic microenvironment in livers of MMP-9–deficient mice. *Manuscript in revision, Cancer Research*

XII. PATENTS

1. Patent Application, *Novel Indications for Transforming Growth Factor-Beta Regulators*. Inventors: **Lisa M. Coussens** and Zena Werb, International filing date: August 9, 2004. Application Filing Number: 60/493,643; Docket Number: 23540-09361/PCT; International Publication Number WO 2005/013915 A2; International Application Number: PCT/US2004/025902; US Application serial Number 10/567,873

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Review Articles, Symposium Proceedings and Editorials

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Humoral immunity, inflammation and cancer

Ting-Ting Tan[1] and Lisa M Coussens[1,2,3]

Clinical and experimental data now clearly indicate that chronic inflammation significantly contributes to cancer development. Emerging out of these studies is an appreciation that persistent humoral immune responses exacerbate recruitment and activation of innate immune cells in neoplastic microenvironments where they regulate tissue remodeling, pro-angiogenic and pro-survival pathways that together potentiate cancer development. Population-based studies examining individuals with chronic inflammatory disorders have revealed that states of suppressed cellular immunity, in combination with enhanced humoral immunity and humoral immunity-associated cytokines, cooperate and effectively suppress anti-tumor immune responses while simultaneously enhancing angiogenesis and presumably overall cancer risk in afflicted tissue. In addition, studies in transgenic mouse models of *de novo* organ-specific cancer development have revealed that inflammation mediated by immunoglobulins and immune complexes might be functionally significant parameters of tumor promotion and progression. These recent advances support the hypothesis that enhanced states of local humoral and innate immune activation, in combination with suppressed cellular immunity and failed cytotoxic T cell anti-tumor immunity, alter cancer risk and therefore represent powerful targets for anti-cancer immunotherapeutics.

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Introduction

Early and persistent inflammatory-type responses in or around developing neoplasms are thought to regulate many aspects of tumor development [1]. The innate immune system, extensively studied in the context of autoimmune disease and wound healing following pathogen infection or tissue damage, has only recently been revealed as an important regulator of cancer development [2,3]. By contrast, tumor immunologists have long focused on anti-tumor activities of the adaptive immune system, and as such have investigated utility of anti-tumor immunotherapeutics with which to combat neoplastic disease [1]. When considering the adaptive immune system as a therapeutic tool, however, it is important to consider both humoral immunity (HI) and cell-mediated immunity (CMI).

This review focuses on the role of persistent humoral-mediated inflammatory responses associated with tumor development and examines the molecular pathways they activate that might differentially regulate cancer promotion and/or progression.

Imbalances in humoral and cell-mediated immunity are associated with cancer development

Pre-malignant and malignant tissues are known to be associated with alterations in immune cell functions (Table 1). Such alterations include suppressed CMI, associated with failure to reject tumors, in combination with enhanced HI that can potentiate tumor promotion and progression [4]. Distinctive $CD4^+$ T-cell subsets (e.g. Th1 or Th2 T helper cells) secrete unique repertoires of cytokines that mediate their responses. Th1 cells produce interleukin (IL)-2 and interferon (IFN)-γ for example, and therefore direct CMI responses, whereas Th2 cells produce IL-4 and IL-10, for example, and facilitate local HI responses. In peripheral blood of patients with bladder and colorectal cancer, proportions of Th1 cells, identified by intracellular production of IFNγ or IL-2, is markedly reduced, whereas proportions of Th2 cells producing IL-4, IL-6 and/or IL-10 is significantly elevated, as compared with proportions of Th1 and Th2 in otherwise healthy patient populations [5,6]. A recent study investigating characteristics of leukocytic infiltrations within colorectal cancers found that CD3+ T lymphocyte densities within tumor biopsies, as opposed to peripheral blood, represented a better predictor of patient survival than current histopathological staging methods [7••]. Moreover, in human cervical carcinomas, CD3+ tumor infiltrating T cells display enhanced Th2 cytokine profiles, specifically increased IL-4 and reduced IFN-γ production [8].

In keeping with these findings, alterations in immune cell status (suppressed CMI and enhanced HI) have also been reported in chronic inflammatory diseases associated with increased cancer risk (Table 1) [4,9,10,11•,12,13]. For

Table 1

Enhanced humoral immunity in pre-malignant and malignant diseases.

Disease state	Reported alteration in immune status/function	References
Malignant tissue		
Bladder cancer	Decreased number of Th1 cells and increased number of Th2 cells in peripheral blood	[5]
Colorectal cancer	Decreased number of Th1 cells and increased number of Th2 cells in peripheral blood and increased Th2 cytokines in serum	[6]
Cervical cancer	Lymphocytes derived from human cervical cancer tissue consisted mainly of Th2/Tc2 phenotypes	[8]
Gastric cancer	Decreased ratio of Th1 cell to Th2 cell in peripheral blood and increased IL-10 in serum	[6,22•]
Head and neck cancer	High level of immune complexes correlates with increased tumor burden and poor prognosis	[34–36]
Breast cancer		
Genitourinary		
Pre-malignant tissue		
Ulcerative colitis	B cell activation and markedly skewed local IgG_1 response in intestines	[4,9]
Asbestosis	Increased immunoglobulin and immune-complex, and higher levels of IL-6 and IL-8 in the peripheral blood of patients with asbestosis	[4,10]
Barrett's esophagus	Replacement of Th1 effector cells with Th2 effector cells in inflamed tissue	[11•]
HCV-related cirrhosis	Decreased number of Th1 cells and increased number of Th2 cells in peripheral blood	[4,12]
Chronic obstructive airway disease (COPD)	T lymphocytes in bronchoalveolar lavage from patients with COPD displayed increased intracellular expression of Th2 cytokines	[4,13]

example, intestinal B cell responses have been observed in ulcerative colitis, a benign condition with a high risk for colorectal cancer development [4,9]. Decreased Th1/Th2 ratios in peripheral blood have been reported in Hepatitis C virus-related liver cirrhosis, a liver disease closely associated with hepatocellular carcinoma [12]. In Barrett's esophagus, an intermediate step in the progression from reflux esophagitis to esophageal adenocarcinoma, infiltration of Th1 effector cells (macrophages and $CD8^+$ T cells) is largely replaced by Th2 effector cells (IgG producing plasma cells and mast cells) when reflux esophagitis progresses to Barrett's esophagus [11•]. Taken together, these compelling clinical findings indicate that pronounced HI may underlie increased risk for neoplastic progression in tissues afflicted with chronic inflammatory disease pathologies.

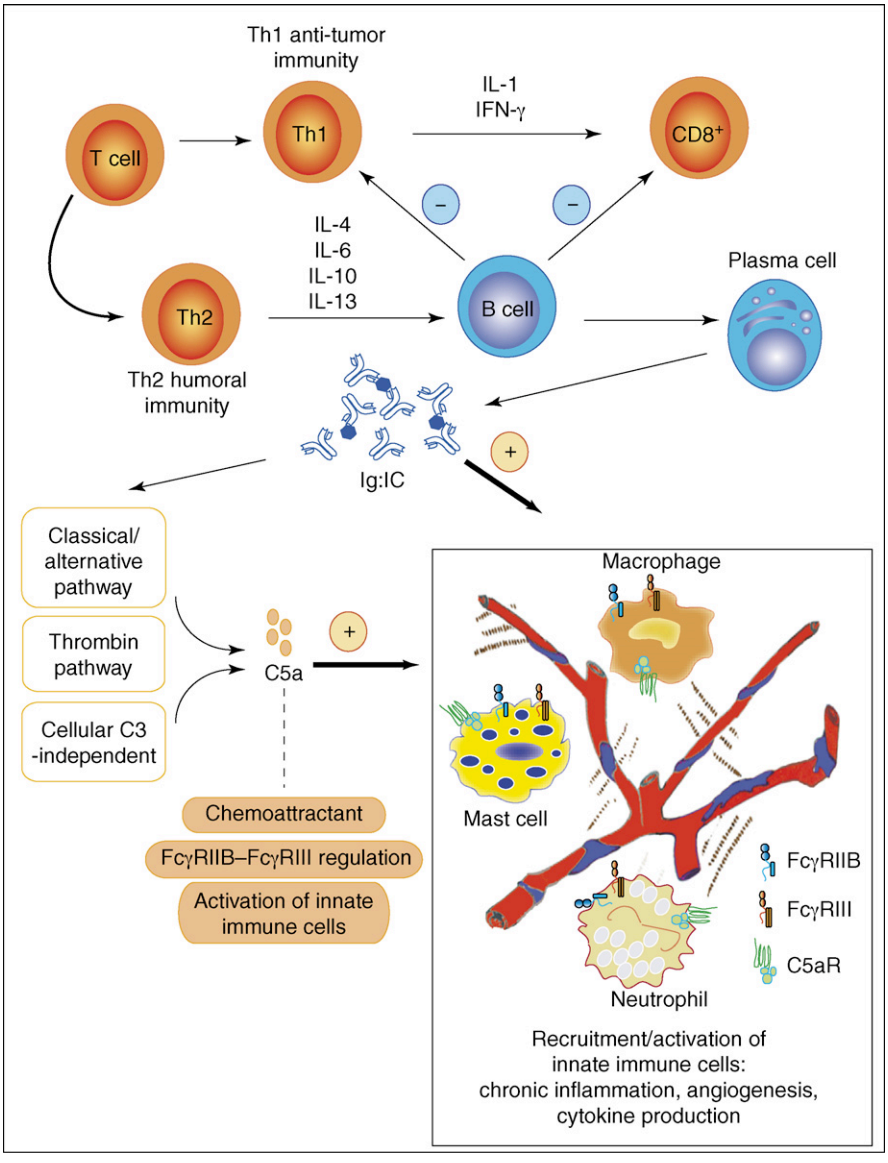
Autoimmune disorders caused by B cell hyperactivity are also associated with cancer [14]. B cells are known to initiate autoimmunity through several mechanistic pathways including enhanced production of autoantibodies, immune complexes, dendritic and T cell activation and cytokine production [15]. The pathogenic role for B cells in autoimmune disease is supported by clinical success of B cell depletion therapy using a chimeric monoclonal antibody (MoAb) specific for human CD20 (e.g. Rituximab) in patients with rheumatoid arthritis, systemic lupus erythematosus (SLE) and others [16,17]. Rituximab has also found clinical efficacy in adult acute lymphoblastic leukaemia (ALL) — monotherapy in patients with relapsed ALL has achieved modest success, but greater effects have been found in combination with chemotherapy and in treatment for minimal residual disease [18]. Although Rituximab effectively deletes the vast majority of circulating B cells, no increased susceptibility to infection has been observed in patients treated with Rituximab for rheumatoid arthritis or non-Hodgkin's lymphoma [17], thus engendering support for systemically manipulating humoral immune responses as a therapeutic approach.

The mechanistic links between autoimmune diseases, such as rheumatoid arthritis, Sjogren's syndrome and SLE, with non-Hodgkin's lymphoma are undisputed; however, the association of solid tumors with autoimmunity has not been well described [14]. Cohort studies have found increased risk for lung cancer in rheumatoid arthritis patients [14], and a modestly increased risk for all cancers, particularly lung cancer and hepatobiliary cancers, in SLE patients [14]. Clinical studies of patients with systemic sclerosis have also revealed increased risk for lung cancer, non-melanoma skin cancers and breast cancer [19]. Mechanisms contributing to these enhanced cancer risks are largely elusive; however, given pronounced humoral immune responses in afflicted tissues, it is intriguing to speculate that autoantibody–antigen complex formation and deposition in neoplastic microenvironments might contribute. Support for this hypothesis comes from a limited clinical study where advanced colon cancer patients were treated with Rituximab. In these individuals, numbers of CD21-hyperpositive lymphocytes were reduced in parallel with a 50% reduction in tumor burden with no ill-effects as a result of the therapy [20]. Taken together, the clinical data indicate a role for enhanced HI and inflammation, in combination with suppressed CMI, in the pathogenesis of several human cancer types — mechanistic investigation of the molecular and cellular pathways mediating enhanced cancer risk will surely identify new therapeutic targets with which to combat neoplastic disease.

HI-associated cytokines as mediators of tumor development

Molecular mechanisms by which HI impacts cancer initiation, promotion and progression are almost certainly multifaceted (Figure 1). Cytokines derived from evolving neoplastic cells, activated resident stromal cells or infiltrating immune cells can regulate tumor growth by affecting angiogenesis, cell survival, death or differentiation. CMI and Th1-associated cytokines, such as IFN-γ, tend to exhibit anti-angiogenic bioactivities [21], whereas several HI and Th2-associated cytokines, such as IL-10, stimulate angiogenesis, and are therefore pro-tumorigenic [22•]. Increased IL-10 expression in patients with gastric cancer correlates with tumor angiogenesis, attenuated CD8+ T cell infiltration, and poor prognosis [22•]. Although, the literature has implicated IL-6 in both Th1- and Th2-type responses, in several chronic inflammatory diseases and various types of cancers, IL-6 is better known to direct Th2-type responses and play a central role as a differentiation and growth factor of

Figure 1



Model for role of humoral immunity during inflammation-associated cancer development. Pre-malignant and malignant tissues are associated with suppressed Th1 responses (i.e. IL-1 and IFNγ) in combination with enhanced Th2 responses (i.e. IL-4, -6, -10 and -13). The latter leads to activation of B cells that can inhibit Th1 anti-tumor immunity. Conversely, B cell activation also leads to immune complex (Ig:IC) accumulation in serum and tissue interstitia that subsequently initiates pro-inflammatory activities and recruitment of innate immune cells (e.g. macrophages, neutrophils and mast cells), possibly via C5aR and FcγRs, leading to chronic tumor-promoting inflammatory responses. C5a, generated through classical/alternative, thrombin and/or cellular C3-independent pathways, induces chemotaxis of innate immune cells and modulates their function via regulation of activating versus inhibitory FcγRs.

neoplastic epithelial cells [23,24]. IL-13 promotes survival and/or growth of selective tumor types through direct action on neoplastic cells, in addition to suppressing CMI [25]. IL-23, a cytokine produced by dendritic cells and macrophages following bacterial exposure and Toll-like receptor engagement, is also found highly expressed in various types of human carcinomas compared with adjacent normal tissue, indicating a potential important role in tumor development [26]. In a mouse model of chemical carcinogenesis, absence of IL-23 resulted in a significant reduction in local inflammatory responses in the tumor microenvironment that paralleled an increase in cytotoxic T-cell infiltration, together resulting in resistance to carcinogenesis [26]. Thus, whereas IL-23 is not generally considered as a Th2 cytokine, in this context, it is exerting Th2-like cytokine bioactivities by promoting inflammatory responses and inhibiting cytotoxic T cell responses. The degree to which effectiveness of IL-23 neutralization will translate to other mouse models of *de novo* carciogenesis and/or human cancer development is yet to be defined.

Thus, Th1- and Th2-associated cytokines act antagonistically in different tumor microenvironments, while in general a cytokine profile skewed toward Th2 responses correlates with enhanced tumor promotion and progression. It is unclear at present, however, if pro-tumor bioactivities of Th2-associated cytokines are limited solely to their direct abilities to enhance pro-tumor pathways (inflammation or angiogenesis), or more in their ability to suppress CMI responses, or a combination of both.

B-lymphocytes inhibit Th1 anti-tumor immunity

As the central component of HI, B-lymphocytes function in antibody production, antigen presentation and secretion of pro-inflammatory cytokines. In the context of cancer development, in addition to altering local and circulating profiles of cytokines, B cells also inhibit Th1-mediated anti-tumor immune responses.

In a syngeneic mouse mammary xenograft model, partial B cell depletion resulted in significantly reduced tumor burden [20]. In experimental mouse models of lung adenocarcinoma, B cell deficiency significantly enhanced therapeutic efficacy of combinatorial chemotherapy and IL-15, a Th-1 cytokine with IL-2-like anti-tumor bioactivities [27]. In a mouse model of melanoma, B cell deficiency provided a therapeutic advantage to a melanoma vaccine where enhanced tumor protection in the absence of B cells was associated with an increased magnitude and longevity of specific cellular immune response provoked by vaccination [28]. Moreover, Shah and colleagues [29•] have reported that B-cell-deficient mice exhibit resistance to several histologically diverse primary syngeneic tumors. Increased tumor resistance in B-cell-deficient mice was found to be associated with enhanced anti-tumor Th1 cytokines and cytolytic T lymphocyte (CTL) responses [29•]. Adoptive transfer of B lymphocytes, but not serum, from wild-type mice to B cell-deficient mice restored tumor growth accompanied by reduced Th1 cytokines and CTL response, suggesting antibody-independent mechanisms [29•]. Together, these experimental findings support the concept that B cells limit anti-tumor immunity by inhibiting Th1 and CTL responses while simultaneously bolstering Th2-effector cell pro-tumor functions.

Do immunoglobulins play a functional role in solid tumor development?

Cancer patients often develop antibodies to tumor-associated antigens — evidence exists to support this for c-myc, HER-2/neu and p53 [30]. However, production of these antibodies does not confer protection, but, paradoxically, correlates with poor prognosis and decreased survival for several human cancer types [31]. Anti-tumor antibodies are thought to enhance tumor growth by promoting pro-tumor immune responses and in general protecting tumor cells from CTL-mediated killing [32]. Increased levels of immunoglobulins (Ig) in neoplastic microenvironments also result in accumulation of immune complexes (ICs) that engender tumor-promoting inflammatory responses [1,33]. Ig-IC formation is a significant feature of cancer development: high circulating levels of ICs are associated with increased tumor burden and poor prognosis in patients with breast, genitourinary, and head and neck malignancies [34–36], and Ig deposition in neoplastic stroma has been reported in pre-malignant and malignant human breast and prostate tissues [1]. McDonald and colleagues [37] recently reported that deposition of extravasated Ig in tumor stroma is due to enhanced leakiness of tumor blood vessels. Using a mouse model of islet cell carcinogenesis, non-specific and epitope-specific antibodies (directed against extracellular matrix antigens) were infused into tumor-bearing animals and both were found to preferentially localize adjacent to leaky tumor blood vessels in tumor stroma [37], thus implying that Ig-IC depositions in neoplastic tissues is a general phenomenon associated with cancer development and not necessarily a directed process towards specific antigens, even when specific antigens are present.

In addition to forming immune complexes, IgG has also been identified in complex with latent transforming growth factor (TGF)-β [38]. Such complexes were proposed to diminish directed primary CTL responses but underlying mechanisms were not fully resolved. TGFβ is a potent regulator of CTL function where it inhibits secretion of cytolytic factors [39]. Kim and colleagues [40••] recently reported that genetic deficiency of key components of the TGF-β intracellular signaling cascade (e.g. *Smad4*) in $CD4^+$ T cells significantly influenced initiation, promotion and progression of epithelial cancer. Mice harboring homozygous null deletions in the *Smad4* gene preferentially in $CD4^+$ T cells spontaneously

develop epithelial cancers in the gastrointestinal tract where prominent plasma cell hyperplasia was evident adjacent to developing duodenal polyps. Mice harboring germline mutations in *Smad4* did not exhibit any of these same characteristics [40••]. *Smad4*-deficient T cells become skewed in their cytokine profile and prominently express several Th2-type cytokines including IL-4, -5, -6 and -13. These pro-tumor mediators also enhance plasma cell differentiation, thus demonstrating that altering local balances of important cytokine pathways, by differential regulation of either HI or CMI, profoundly influences malignant risk and argues that perturbing these balances to favor anti-tumor immunity represents a powerful anti-cancer approach.

ICs have long been suspected as initiators of inflammatory cascades associated with tissue destruction in autoimmune diseases but underlying molecular mechanisms have been elusive [41]. Recent investigations of these mechanisms using an experimental mouse model of airway remodeling following *Mycoplasma pulmonis* infection have demonstrated that peripheral B cell responses and local Ig-IC deposition are the critical triggers for recruiting innate leucocytes into infected airways, that then activate pro-angiogenic and tissue remodeling pathways necessary for resolving infection [42]. We have reported complimentary findings using a transgenic mouse model of squamous carcinogenesis [43,44•]. Here, oncogenes from human papillomavirus type 16 (HPV16) are expressed in skin keratinocytes under direction of a human keratin 14 (K14) promoter/enhancer and initiate multi-stage development of squamous cell carcinomas (SCCs) in skin and cervix [43,44•,45]. In HPV16 transgenic mice, combined B- and T-lymphocyte-deficiency eliminated IC deposition in premalignant skin and attenuated innate immune cell infiltrations, resulting in diminished tissue remodeling activity, failure to activate angiogenic vasculature, retention of terminal differentiation programs in skin keratinocytes and a 43% reduction in overall SCC incidence [44•]. Adoptive transfer of B-lymphocytes or serum isolated from HPV16 mice (but not from wild-type naïve mice) into B- and T-lymphocyte-deficient/HPV16 mice restored IC deposition, chronic innate immune cell infiltration in pre-malignant skin and reinstated parameters for full malignancy [44•]. These data indicate that B-lymphocyte-derived factors, possibly Ig, are essential for establishing chronic inflammatory pathways that potentiate cancer development. In support of this concept, anti-tumor antibodies are known to enhance outgrowth and invasion of murine and human tumor-cell xenografts through recruitment and activation of granulocytes and macrophages [33], which are important sources of vascular endothelial growth factor (VEGF) [46] that possesses pro-angiogenic bioactivities. Thus, serum proteins (presumably antibodies) produced by B-lymphocytes locally or peripherally, at least in some scenarios, are crucial factors that initiate chronic inflammatory programs, that are not only essential for resolving acute tissue damage but also essential for promoting tumor development in initiated tissues.

FcγR and C5a are key regulators of IC-mediated inflammation

Inflammatory responses to ICs have been extensively studied in transgenic mouse models of various autoimmune diseases [47]. Mechanisms by which ICs initiate inflammation are still not fully understood; however, receptors for the Fc portion of IgG (FcγRs, especially FcγRIII) and complement factors (particularly C5a anaphylatoxin) are recognized as co-dominant effectors in the process [47]. Considering the fact that tissues damaged by autoimmune dysfunction and by cancer have similar characteristics (e.g. chronic innate immune cell infiltration, tissue remodeling, angiogenesis, altered cell survival pathways) [48], it seems probable that similar IC effector pathways are involved in pathogenesis of both diseases.

Expressed on most leucocytes, FcγRs are classified into four groups (FcγRI/CD64, FcγRII/CD32, FcγRIII/CD16 and recently identified FcγRIV) according to their distinct affinities for IgG, cell distributions and functions. FcγRI and FcγRIII, predominantly FcγRIII, mediate immune cell activation via their FcR γ chain that contains an intracellular tyrosine-based activating motif (ITAM). Activating signals mediated by ITAM trigger oxidative bursts, cytokine release and phagocytosis by macrophages, antibody-dependent cell-mediated cytotoxicity (ADCC) by natural killer (NK) cells, and degranulation of mast cells [41]. In contrast, engagement of FcγRIIB that instead contains immune tyrosine-based inhibitory motifs (ITIM) inhibits these same inflammatory responses [41]. These two opposing regulatory pathways coexist on innate immune cells and thus, in part, determine the magnitude of IC-mediated inflammatory responses. Indeed, the ratio of activating to inhibitory FcγR is low in 'normal' homeostatic tissues, and by contrast is highly increased in inflamed microenvironments [47].

Receptors for the Fc portion of IgG, FcγRs play a central role in regulating immune responses following interaction with ICs [41]. It was recently demonstrated that presence or absence of sialic acid at the terminus of the core glycan in the Fc region of IgG regulates immune response [49••]. Sialylation of the Fc region of IgG reduces binding affinities towards FcγRs thereby inhibiting pro-inflammatory activities of IgG, whereas reduction of sialylation, upon antigen challenge, switches the immune response pathway from anti-inflammatory to pro-inflammatory via differential engagement with FcγRs on effector cells [49••]. Regulatory functions of FcγRs have been studied using genetically engineered animal models. Mice deficient in the FcR γ chain or activating type FcγRs are resistant to a wide range of IC-mediated hypersensitive reactions, such as vasculitis, glomerulonephritis and skin Arthus reaction

[41]. Conversely, mice deficient in FcγRIIB exhibit enhanced IC-mediated inflammatory responses [41]. Although these elegant genetic studies demonstrate functional importance of FcγRs in IC-mediated inflammation and autoimmune diseases, little is known about their role in tumor development.

Complement activation is a central event during innate immune defence following pathogenic tissue assault. Three different pathways of complement activation have been identified, namely classical, alternative and lectin pathways. Foreign antigens and ICs activate complement cascades resulting in formation of lytic membrane attack complexes and formation and liberation of anaphylatoxins; for example, C3a and C5a, potent pro-inflammatory factors that induce recruitment and activation of leukocytes. Deposition of complement proteins is a common occurrence at sites of inflammation. Studies utilizing complement depleted mice and C3- or C5-deficient mice have identified their crucial roles in regulating disease pathogenesis [50,51]. We previously reported abundant deposition of C3 in neoplastic skin of HPV16 mice; but found that C3-deficiency was without functional consequence, indicating that if Ig-ICs enhance premalignant progression, they most likely contribute via regulation of either FcγR or through C5a [52].

C5a is a proteolytic fragment of C5 generated by the high-affinity C5 convertase, C3b complexed with C4b and C2a. Of note, complement-independent enzymes, such as thrombin, neutrophil elastase and a macrophage serine protease, have C5 convertase activity and therefore generate C5a in a C3-independent manner [53•] (Figure 1). C5a induces chemotaxis of macrophages, granulocytes, mast cells, dendritic cells and B-lymphocytes, and modulates their function through differential regulation of activating FcγRs versus inhibitory FcγRs [54•]. The initial contact between ICs and pulmonary alveolar macrophages results in complement-independent C5a production that causes decreased levels of inhibitory FcγRIIB, increased levels of activating FcγRIII, and highly induced FcγR-mediated cytokine production [54•] (Figure 1). Whether C5a-dependent modulation of FcγRs is a common mechanism operative in IC inflammation has yet to be determined. Nevertheless, a cellular pathway of C5a production is evident in cultured macrophages induced by antibody complex ligation to activating FcγR, indicating a complex regulatory network of multiple interactions between FcγR and C5a/C5aR [47]. Considering that autoimmune diseases and cancer have common characteristics such as pronounced HI and IC deposition, FcγR and C5a/C5aR are likely to play a role in cancer-associated inflammation.

Conclusions

Both clinical data and experimental animal studies indicate that sustained humoral immune responses initiate significant pro-tumor effects on developing neoplasms. We propose that bolstering anti-tumor cell-mediated immune responses, in combination with neutralizing potent pro-tumor humoral immune responses with efficacious immuno-therapeutics, would effectively direct tumor suppression while establishing an immune environment favoring therapeutic responses to conventional anti-tumor modalities. Certainly, further studies are needed to determine the universality of this approach and specifically which types of human cancers would be amenable to such therapy. Nonetheless, a dedicated attempt to reveal the molecular and cellular mechanisms linking HI, inflammation and cancer will also provide important insights into viable therapeutic targets for anti-cancer therapy.

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Immune Cells as Anti-Cancer Therapeutic Targets and Tools

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Abstract Chronic inflammation is a contributing factor to overall cancer risk as well as cancer promotion and progression; however, pathways regulating onset of cancer-promoting inflammatory responses are still poorly understood. Clinical data suggest that deficient anti-tumor cell-mediated immunity, in combination with enhanced pro-tumor humoral and/or innate immunity (inflammation), are significant factors influencing malignant outcome. Here, we discuss therapeutic implications from clinical data and experimental studies using de novo immune-competent mouse models of cancer development that together are revealing molecular and cellular mechanisms underlying interactions between immune cells and evolving neoplastic cells that regulate cancer outcome. Understanding the functionally significant links between adaptive and innate immunity that regulate cancer development will open new therapeutic opportunities to manipulate aspects of immunobiology and minimize lethal effects of cancer development. J. Cell. Biochem. 101: 918–926, 2007.

Key words: cancer; immune cells; inflammation; innate; adative

In the past 25 years, a majority of cancer studies have focused on examining functional consequences of activating and/or inactivating mutations in critical genes implicated in cell cycle control. While these studies have been instructive regarding the role of oncogene and tumor suppressor gene functions and signaling pathways regulating cell proliferation and/or cell death, they have largely ignored the fact that in vivo, cancers are heterogeneous multicellular growths whose survival and dissemination is dependent upon reciprocal interactions between genetically modified "initiated" cells and a dynamic microenvironment in which they live. Cancers are composed of multiple cell types, for example, fibroblasts, epithelial cells, innate and adaptive immune cells, cells forming blood and lymphatic vasculature, as well as specialized mesenchymal cell-types unique to each tissue microenvironment. While tissue homeostasis is maintained by collaborative interactions between all of these distinct cell types, cancer development is enhanced when mutant cells harness these collaborative capabilities to favor their own survival. Thus, genomic alterations effecting intrinsic cellular programs, for example, cell cycle check-point control, programmed cell death, differentiation, metabolism and cell adhesion, in combination with somatic or epigenetic alterations effecting extrinsic programs such as immune response, matrix metabolism, tissue oxygenation, and vascular status, underlie human cancer development.

IMMUNE REGULATION OF TISSUE HOMEOSTASIS

The mammalian immune system consists of multiple cell types and mediators that interact with each other and non-immune cells in complex and dynamic networks to ensure protection against foreign pathogens, while

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simultaneously maintaining tolerance towards self-antigens. Based on antigen specificity and timing of activation, the immune system is composed of distinct subsets—adaptive and innate. While cellular composition and antigen specificity of these subsets are distinct, each has developed sophisticated communication networks enabling rapid responses to foreign antigens.

Innate immune cells, for example, dendritic cells (DC), natural killer (NK) cells, macrophages, neutrophils, basophils, eosinophils, and mast cells, are first lines of defense against tissue injury. DCs, macrophages and mast cells, serve as sentinel cells pre-stationed in tissues and monitor their microenvironment continuously for signs of distress. When tissue homeostasis is disturbed, sentinel cells release soluble mediators (cytokines, chemokines, matrix remodeling proteases, reactive oxygen species (ROS), and bioactive mediators, e.g., histamine) that together induce mobilization and infiltration of additional leukocytes into damaged tissue (i.e., inflammation). Macrophages and mast cells also activate vascular and fibroblast responses in order to orchestrate elimination of invading organisms and initiate local tissue regeneration. DCs on the other hand, take up foreign antigens and migrate to lymphoid organs where they present their antigens to adaptive immune cells, thus acting as key players in the interface between innate and adaptive immunity. NK cells also participate in cellular crosstalk between innate and adaptive immune cells via their ability to bidirectionally interact with DCs; certain NK cell subsets eliminate immature DCs, whereas others stimulate DC maturation that then also reciprocally regulate NK cell activation [Raulet, 2004; Degli-Esposti and Smyth, 2005; Hamerman et al., 2005].

Induction of efficient primary adaptive immune responses requires direct interactions with mature antigen presenting cells and a pro-inflammatory milieu. Adaptive lymphocytes, such as B cells, $CD4^+$ (helper) and $CD8^+$ cytotoxic T lymphocytes (CTL), distinguish themselves from innate leukocytes by expression of somatically generated, diverse antigen-specific receptors, formed through random gene rearrangements, allowing a flexible and broader repertoire of responses as compared to innate immune cells expressing germline-encoded receptors. Distinctive $CD4^+$ T-cell subsets, for example, Th1 or Th2 T helper cells, secrete unique repertoires of cytokines that mediate their responses. Th1 cells produce interleukin (IL)-2 and interferon (IFN)-γ for example, and thereby direct cell-mediated immune (CMI) responses, whereas Th2 cells secrete IL-4 and IL-10 and facilitate local humoral immune (HI) responses. Together, activation of innate and adaptive immune response pathways efficiently removes or eliminates invading pathogens, damaged cells and extracellular matrix (ECM). Once assaulting agents are eliminated, immune cells are critically involved in normalizing cell proliferation and cell death pathways to enable re-epithelialization, new ECM synthesis and re-establishment of tissue homeostasis.

ANTITUMOR ACTIVITIES OF IMMUNE CELLS

The role of the immune system is to protect the body against infectious agents and to facilitate healing process following injury. Therefore, it seems intuitive that immune cells would also play an active role protecting against primary tumor development and/or metastases. Indeed, individuals suffering from various types of immune-deficiency disorders exhibit increased risk for some viral- and/or carcinogen-associated cancers [Zitvogel et al., 2006], thus indicating that absence of anti-viral immunity has effected their relative cancer risk. On the other hand, the relative risk of common epithelial cancers such as breast, prostate, ovarian, and uterine cancer, where cancer etiology is not commonly associated with viral infection or carcinogen exposure, is less than 1.0 in similar cohorts [de Visser et al., 2006], thus indicating a paradoxical regulatory role for the immune system during cancer development where cancer etiology is key.

Lymphocytes and some innate immune cells possess potent anti-cancer activities that can effect growth and/or dissemination of primary tumors. A recent study investigating characteristics of leukocytic infiltrations within colorectal cancers found that $CD3^+$ T cell densities within colorectal cancer biopsies, as opposed to peripheral blood, represented a better predictor of patient survival than current histopathological staging methods [Galon et al., 2006]. Infiltration of NK cells in human gastric or colorectal carcinoma is similarly associated with a favorable prognosis [Coca et al., 1997]. The major anti-cancer function of NK cells likely owes to

their ability to eliminate neoplastic cells with downregulated human leukocyte antigen (HLA) expression before they acquire malignant characteristics. The most compelling evidence for involvement of NK cells in killing human tumor cells in vivo derives from allogeneic bone marrow transplantation, where data indicates their ability to lyse tumor cells ex vivo, presence of NK cells within tumors, increased NK cell function and anti-tumor response in individuals treated with interleukin (IL)-2 and the correlation of decreased NK cell function with tumor progression [Orange and Ballas, 2006].

Based on the idea that a "tumor" can be a recognizable target for the adaptive immune system, several groups have attempted to activate adaptive immune cells in order to elicit anti-tumor immune responses [Dudley and Rosenberg, 2003]. In several experimental murine tumor models, $CD8^+$ T cells were found to be required for antitumor effects [Zitvogel et al., 2006]. Furthermore, that cytotoxic T-cells were able to eliminate only tumor cells expressing their cognate antigen, indicates a specific immune response [Dudley et al., 2003]. Interestingly, treating (by adoptive transfer) animals with tumor-associated B cells has been reported to result in the opposite effect, stimulating tumor invasion and metastasis through antibody–antigen complex-mediated granulocyte and macrophage induction [Barbera-Guillem et al., 1999], and thus highlighting the need to more fully understand all components of adaptive immunity activated during cancer development in tissues.

In order to survive, neoplastic cells must evade cytotoxic T lymphocyte rejection. This can be achieved through subversion of host anti-tumor immune responses. One plausible explanation for how tumor cells escape immune surveillance mechanisms is that neoplastic microenvironments favor polarized chronic pro-tumorigenic inflammatory states as opposed to those representing acute anti-tumor immune responses [Balkwill et al., 2005; Zou, 2005]. Clinical data indicate that the "immune status" of healthy individuals as compared to those harboring malignant tumors is distinct, where in the later population, T lymphocytes are found to be functionally impaired [Finke et al., 1999]. In addition, accumulation of chronically activated granulocytes/suppressor cells and regulatory T cells are found in the circulation, in lymphoid organs and in neoplastic tissues [Curiel et al., 2004; Serafini et al., 2004]. Together, immune states such as these disable tumor-killing $CD8^+$ CTL responses and enable states of immune privilege that foster escape from anti-tumor immunity while simultaneously exploiting activated immune cells that enhance cancer development.

Chronically activated innate immune cells can indirectly contribute to cancer development via suppression of anti-tumor adaptive immune responses, allowing tumor escape from immune surveillance. A subset of innate immune cells, for example, myeloid suppressor GR^+CD11b^+ cells, accumulate in peripheral blood of cancer patients [Almand et al., 2001; Serafini et al., 2004], as well as in tumors and lymphoid organs [Gabrilovich et al., 2001; Serafini et al., 2004; Zou, 2005]. Myeloid suppressor cells are known to induce T lymphocyte dysfunction by direct cell–cell contact and by production of immunosuppressive mediators, and thus actively inhibit anti-tumor adaptive immunity [Gabrilovich et al., 2001; Serafini et al., 2004]. Myeloid suppressor cells can also directly promote tumor growth by contributing to tumor-associated angiogenesis [Yang et al., 2004]. In addition, malignant lesions attract regulatory T cells that can suppress effector functions of cytotoxic T cells [Zou, 2005]. Classic regulatory T cells are $CD4^+CD25^+FOXP3^+$, however, different subtypes may also exist. Initial investigations have revealed that in vivo depletion of regulatory T cells using antibodies against CD25 enhanced anti-tumor T cell responses and induced regression of experimental tumors [Onizuka et al., 1999; Shimizu et al., 1999]. In an elegant study by Curiel et al. [2004], it was revealed that tumor-derived macrophages from patients with ovarian cancer produce CL22, a chemokine that mediates trafficking of regulatory T cells to tumors. These regulatory T cells in ovarian cancer patients suppressed tumor-specific T cell immunity, and their presence correlated with reduced survival. Thus, in the vicinity of a growing neoplasm, the balance between innate and adaptive immunity is often disturbed in favor of cancer progression. Taken together, the accumulated data from human and animal studies support the existence of an immune response involving $CD8^+$ T cells, T_H1 cells and NK cells that protect against tumor development and progression—a system that can be suppressed locally by myeloid suppressor cells and regulatory T cells.

PROTUMOR ACTIVITIES OF IMMUNE CELLS

The association of immune cells and cancer has been known for over a century [Balkwill and Mantovani, 2001]. Initially, it was believed that leukocytic infiltrates in and around developing neoplasms represented an attempt of the host to eradicate neoplastic cells, as described above. However, clinical and experimental data now indicate that chronic presence and activation of some innate immune cell types, for example, neutrophils, macrophages, and mast cells, exerts a promoting role during cancer development [Coussens and Werb, 2002; Balkwill et al., 2005; de Visser et al., 2006]. Malignant tissues containing infiltrates of macrophages (human breast carcinoma) and mast cells (human lung adenocarcinoma and melanoma), for example, correlate with an unfavorable clinical prognosis [Leek et al., 1996, 1999; Imada et al., 2000; Ribatti et al., 2003]. In experimental murine models of organ-specific cancer development, genetic elimination of mast cells or macrophages minimizes squamous carcinogenesis [Coussens et al., 2001; Giraudo et al., 2004], whereas elimination of macrophages during mammary carcinogenesis limits late-stage cancer progression and pulmonary metastasis formation [Lin et al., 2001]. Other cells of the myeloid lineage also have been reported to contribute to tumor development [Sparmann and Bar-Sagi, 2004]. NK cells can play a role in protection against experimental tumor growth, in part by producing mediators with anti-angiogenic properties [Smyth et al., 2001; Hayakawa et al., 2002]. Together, these studies have induced a paradigm shift regarding the role of immune cells during malignant progression. Whereas the historical viewpoint was that host immunity is protective with regards to cancer, it is now clear that certain subsets of chronically activated innate immune cells promote growth and/or facilitate survival of neoplastic cells.

In support of these experimental findings are population-based studies reporting that chronic inflammatory conditions predispose humans to certain cancers, most notably patients with chronic *Helicobacter pylori* infection exhibit a 75% increased risk for gastric cancer, the second most common type of cancer globally [Ernst and Gold, 2000; Kuper et al., 2000]. Consistent with this are experimental findings demonstrating that development of colon cancer in transforming growth factor beta-1 (TGFβ1)-deficient mice is essentially eliminated by maintaining mice in germ-free environments [Engle et al., 2002]. Other clinical examples where chronic inflammation has an associated increased cancer risk are inflammatory bowel syndrome with colon cancer [Shacter and Weitzman, 2002], chronic pancreatitis with pancreatic adenocarcinoma [Farrow and Evers, 2002], and hepatitis with hepatocellular carcinoma [Shacter and Weitzman, 2002]. Population-based studies examining long-term usage of anti-inflammatory therapeutics, for example, aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), and cycloygenase-2 (COX-2) inhibitors, support the conclusion that chronic inflammation enhances cancer risk [Peek et al., 2005; Ulrich et al., 2006]. However, it must also be appreciated that all organ systems are not identical and as such, there are also data indicating an increased risk of pancreatic cancer and Non-Hodgkin's lymphoma amongst long-term salicylic acid users [Cerhan et al., 2003; Schernhammer et al., 2004].

Innate immune cells directly potentiate cancer risk through the diversity of bioactive mediators they secrete and/or deliver to neoplastic tissue microenvironments. Leukocytes are variably loaded with chemokines, cytokines, cytotoxic mediators including ROS, serine-, cysteine-, and metallo-proteases, membrane-perforating agents, and soluble mediators of cell killing, such as tumor necrosis factor-alpha (TNF-α), interleukins and interferons [Tlsty and Coussens, 2006]. Individually, all of these molecules are known mediators of acute inflammation and evoke innate immune cell recruitment and/or activation, tissue remodeling and angiogenesis, and together, create an organ microenvironment favoring cell proliferation, genomic instability, and expansion of cell populations into ectopic tissue microenvironments, that is, malignant conversion and cancer development. Thus, clinical and experimental data largely indicate a promoting role for innate immune cells during neoplastic progression and suggest that elucidating the mechanisms by which inflammatory cells participate in carcinogenesis may eventually facilitate development of novel anti-cancer therapeutic agents.

Based on the inter-relationship between adaptive and innate immunity in tissue homeostasis and disease [Hoebe et al., 2004], we investigated whether activation of adaptive

immune responses was a critical regulator of chronic inflammation-associated with epithelial cancer development. To achieve this, we generated HPV16-expressing transgenic mice prone to squamous carcinoma development [Coussens et al., 1996], that were genetically deficient (−/−) for recombinase acitivating gene (RAG)-1 and thus lacking all mature B and T lymphocytes [de Visser et al., 2005]. HPV16/RAG-$1^{-/-}$ mice exhibited a markedly decreased infiltration of innate immune cells into premalignant skin that was associated with reduced local levels of tissue remodeling proteases activities and vascular endothelial growth factor (VEGF), lack of activation of angiogenesis, reduced epithelial proliferation, and retention of terminal differentiation in oncogene-positive keratinocytes. Thus, skin of HPV16/RAG-$1^{-/-}$ mice failed to progress beyond a hyperplastic phenotype, resulting in only 6.4% of HPV16/RAG-$1^{-/-}$ mice developing invasive squamous cell carcinomas of the skin as compared to ~50% in control HPV16 mice [de Visser et al., 2005]. Significantly, transfer of B lymphocytes or serum isolated from HPV16 mice, but not naïve wildtype mice, into HPV16/RAG-$1^{-/-}$ mice was sufficient to restore characteristics of premalignant progression, for example, chronic inflammation and infiltration of neoplastic skin by mast cells and neutrophils, development of angiogenic vasculature, epithelial hyperproliferation, and loss of keratinocyte terminal differentiation [de Visser et al., 2005]. Thus, soluble mediators derived from B lymphocytes enhance epithelial carcinogenesis in HPV16 mice by initiating a cascade of chronic inflammation in the premalignant microenvironment.

These experimental data are supported by clinical data revealing presence of antibodies, specific for tumor antigens, in serum of patients with squamous cell carcinoma of the head and neck whose presence were found to correlate to tumor progression and clinical course [Vlock et al., 1992]. Interestingly, an early case study reported that nonspecific removal of serum IgG from a patient with metastatic colon carcinoma correlated with an improvement in the general condition of the patient and decreased tumor size [Bansal et al., 1978]. More recent studies have demonstrated that proportions of T_H1 cells, identified by intracelluar production of interferon (IFN)γ or IL-2 is markedly reduced in peripheral blood of patients with bladder or colorectal cancer, whereas proportions of T_H2 cells producing IL-4, IL-6, and/or IL-10 is significantly elevated, as compared to otherwise healthy patient populations [Kanazawa et al., 2005; Agarwal et al., 2006]. Taken together, the accumulated data from human and experimental animal studies support the existence of a protumor immune response involving B cells, T_H2 cells and activated innate immune cells that favors neoplastic development and emergence of invasive carcinomas.

BALANCING PRO- AND ANTI-TUMOR IMMUNITY TO EFFECT CLINICAL OUTCOME

The Hegelian (or Fichtean) dialectic [Williams, 1992] is often presented in a threefold manner where a thesis is initially provided that gives rise to a reaction, followed by an antithesis, which contradicts the thesis. The conflict is later resolved by formation of a synthesis that reconciles their common truths and forms a new proposition. Perhaps the time has come to coagulate published disparate theories on the role of the immune system in cancer. Thus, there is compelling evidence for the thesis that the immune system protects the organism from tumor development, as well as for its antithesis, that the immune system promotes cancer progression. This paradoxical role during cancer development may seem contradictory; however, the conflict is resolved by altering the way in which the immune systems role is viewed.

During the last decade, insights have been gained regarding mechanisms underlying the dynamic interplay between immune cells and tumor progression. The accumulated data indicates that the outcome of an immune response toward a tumor is largely determined by the type of immune response elicited. A tumor-directed immune response involving cytolytic $CD8^+$ T cells, T_H1 cells, and NK cells appears to protect against tumor development and progression. If, on the other hand, the immune response involves B cells and activation of humoral immunity, and infiltration of T_H2 cells innate inflammatory cells into an organ harboring initiated cells, the likely outcome is promotion of tumor development and progression (Fig. 1). This balance between a protective cytotoxic response and a harmful humoral or T_H2 response can be regulated systemically by the general immune status of the individual, as well as locally by myeloid suppressor cells and

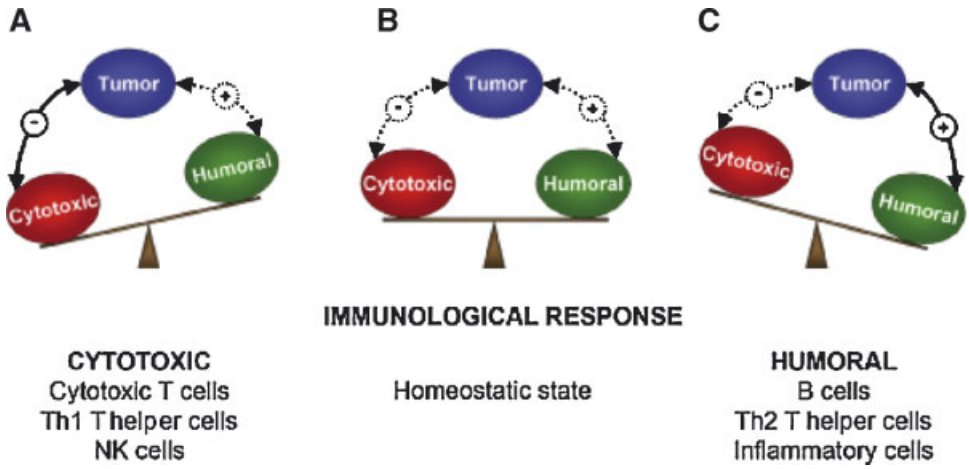

Fig. 1. Schematic overview of dynamic interactions between tumor cells and the host immune system. **A**: Balance in favor of a cytotoxic response, leading to tumor regression, (**B**) tumor evasion of an immunological response, (**C**) balance in favor of a humoral/innate response, stimulating tumor progression. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

regulatory T cells, and thus presents clinicians with attractive targets for anti-cancer immune-based therapies.

HOPES FOR THE FUTURE

Bolstering effective cytotoxic T cell responses, in combination with neutralizing harmful pro-tumor humoral and innate immunity would seem to represent a powerful anti-cancer therapeutic approach. The promise of such an approach recently became apparent in a study by Morgan et al. [2006]. The authors isolated autologous T cells from patients with metastatic melanoma, transfected the cells with a retrovirus encoding a tumor-recognizing T cell receptor, expanded them ex vivo followed by reinfusion into lymphodepleted patients following treatement with IL-2 to stimulate T cell reactivity. Two of 15 patients exhibited durable tumor regression—a promising result demonstrating that bolstering anti-tumor immunity can be an effective clinical tool. However, the low success rate may indicate that achieving robust rejection of solid tumors is limited, perhaps by prominent humoral and/or innate immune mechanisms, even with high numbers of circulating tumor-specific cytotoxic T cells.

Regarding the significance of enhanced humoral immune responses in individuals with cancer, recent data from our laboratory indicates that peripheral B cell activation, in combination with delivery of humoral immune factors to local neoplastic microenvironments, favors carcinoma development [de Visser et al., 2005]. However, the identity of the B cell-derived soluble factor(s) present in serum represent the critical mediators for pro-tumor immunity remains to be determined. Since neoplastic progression in HPV16 mice is characterized by deposition of antibodies in stroma underlying neoplastic epidermis [de Visser et al., 2005] and given the central role of immunoglobulins (Ig) and immune-complexes in regulating several chronic inflammatory diseases [de Visser et al., 2006], we hypothesize that Igs may represent the functional link between peripheral B lymphocyte activation and cancer progression. Continuous presence of antibodies can elicit chronic inflammation via activation of the complement cascade and subsequent cross-linking of complement receptors on resident innate immune cells [Benoist and Mathis, 2002] Alternatively, Igs can induce pro-tumor immune responses following their cross-linking with Fc receptors expressed on innate immune cells [Hogarth, 2002]. Further elucidation of the intrinsic immune cell signaling pathways regulated by enhanced humoral immunity in HPV6 mice may shed light on these mechanisms and reveal potent anti-tumor targets for therapeutic intervention.

Clinical studies also lend support for the concept of B lymphocyte-mediated promotion of cancer development. Many studies have described increased levels of (auto) antibodies in serum or tumors of cancer patients (reviewed in: [de Visser et al., 2005]. Moreover, presence of autoantibodies in serum of breast cancer patients at time of diagnosis correlates with

poor prognosis [Wasserman et al., 1975]. In combination with our data indicating that B lymphocytes exert their pro-tumor effects early during premalignant progression, together suggests that therapies neutralizing B lymphocytes or their downstream effector pathways may represent promising therapeutic targets. Elimination of B lymphocytes by treatment with rituximab, a monoclonal antibody directed against CD20 present on B cells, has now successfully been applied in a variety of autoimmune disorders with relatively few side-effects [Kazkaz and Isenberg, 2004] as well as some hematologic malignancies, where long-term usage appears to be well-tolerated [Hainsworth et al., 2003]. While promising, the efficacy of rituximab, or other reagents neutralizing B cells, remains untested for solid tumors.

Established tumors represent formidable opponents that harbor inherent potential for developing diverse drug resistances. Aside from investing in earlier screening approaches to detect and eradicate premalignant disease, our best hope for minimizing lethal effects of cancer are to develop combinatorial treatment strategies where intrinsic pathways regulating neoplastic cell survival are targeted, in combination with therapies effecting extrinsic pathways that neutralize pro-tumor immunity, bolster anti-tumor immunity and limit or normalize angiogenic blood vessels. Our belief is that a broader understanding of the role of the immune system in tumor development will facilitate development of novel anti-cancer treatment strategies.

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APPENDIX D

Review

Inflammation and breast cancer

Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression

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Abstract

Recent insights into the molecular and cellular mechanisms underlying cancer development have revealed that immune cells functionally regulate epithelial cancer development and progression. Moreover, accumulated clinical and experimental data indicate that the outcome of an immune response toward an evolving breast neoplasm is largely determined by the type of immune response elicited. Acute tumor-directed immune responses involving cytolytic T lymphocytes appear to protect against tumor development, whereas immune responses involving chronic activation of humoral immunity, infiltration by Th2 cells, and protumor-polarized innate inflammatory cells result in the promotion of tumor development and disease progression. Herein we review this body of literature and summarize important new findings revealing the paradoxical role of innate and adaptive leukocytes as regulators of breast carcinogenesis.

Introduction

Breast cancer is the most frequent malignant tumor of women in North America [1]. Standard treatment modalities have improved the overall outlook and quality of life for women with breast cancer; however, the fact that 40% still succumb to disease highlights the need for new therapeutic approaches and identification of new therapeutic targets. While genetic and epigenetic changes in genes that regulate mammary epithelial cell proliferation, survival, polarity and/or differentiation are probable 'initiators' of breast carcinogenesis, several lines of evidence indicate that stromal cell responses in premalignant mammary tissue may 'promote' progression to cancer and/or the metastatic capability of malignant mammary epithelial cells. Cellular components of tumor stroma include (myo)fibroblasts, vascular cells, infiltrating leukocytes and specialized mesenchymal support cells unique to each tissue microenvironment. A growing body of evidence has recently implicated tumor-infiltrating leukocytes as causal players in cancer development [2-8].

The present review focuses on the paradoxical roles of innate and adaptive leukocytes as regulators of breast carcinogenesis, and highlights recent experimental data indicating that therapeutically targeting these diverse immune cell types by either neutralizing and/or bolstering their specific bioactivities may provide a therapeutic advantage to patients with breast cancer.

Leukocytes and carcinoma development

Leukocytes represent a diverse assortment of immune cells composed of both innate (myeloid) and adaptive (lymphoid) lineages. Innate immune cells, including macrophages, granulocytes, mast cells, dendritic cells (DCs), and natural killer (NK) cells, represent the first line of defense against pathogens and foreign agents. When tissue homeostasis is perturbed, tissue-resident macrophages and mast cells locally secrete soluble factors such as cytokines, chemokines, bioactive mediators, and matrix-remodeling proteins that recruit additional leukocytes from the circulation into damaged tissue (that is, inflammation) [3,9,10]. Recruited innate immune cells can directly eliminate pathogenic agents *in situ*. At the same time, DCs take up foreign antigens (including tumor antigens) and migrate to lymphoid organs, where they present their antigens to adaptive immune cells. Upon recognition of foreign antigen presented by DCs or other professional antigen-presenting cells, adaptive immune

CSF = colony-stimulating factor; CTL = cytotoxic T lymphocyte; DC = dendritic cell; DCIS = ductal carcinoma *in situ*; IFN = interferon; IL = interleukin; NK = natural killer; Th cells = T-helper cells; TNF = tumor necrosis factor; T_{reg} cells = regulatory T cells; VEGF = vascular endothelial growth factor.

cells, such as T lymphocytes or B lymphocytes, undergo clonal expansion in order to mount an 'adaptive' response targeted against the foreign agent [11,12]. Acute activation of innate immunity therefore sets the stage for activation of more sophisticated, antigenically committed, adaptive immune responses. Once foreign agents have been eliminated, inflammation resolves and tissue homeostasis is restored.

The inflammatory responses necessary for enabling an immune reaction may, however, also set the stage for promoting neoplastic disease. As early as 1863, Virchow first postulated that cancer originates at sites of chronic inflammation, in part based on his hypothesis that some classes of irritants causing inflammation also enhance cell proliferation [13]. When tissues are injured or are exposed to chemical irritants, damaged cells are removed by the induction of cell death pathways, while cell proliferation is enhanced to facilitate tissue regeneration in an attempt to re-establish tissue homeostasis. Proliferation and inflammation resolve only after insulting agents are removed or tissue repair is completed. In contrast, when insulting agents persist over time, sustained cycles of cell proliferation and death in environments rich in inflammatory cells and their bioactive products may increase neoplastic risk and foster tumor progression [3]. While sporadic or inherited genetic mutations in critical genes regulating cell cycle, programmed cell death, differentiation and adhesion may represent initiating events in tumorigenesis ('initiation'), chronic inflammation favors selection of additional features in initiated cells that may promote their full malignant transition ('promotion').

Historically, leukocytes found in and around developing tumors were thought to represent an attempt by the host to eradicate transformed neoplastic cells. Certain leukocytes, such as cytotoxic T lymphocytes (CTLs) and NK cells, undeniably play a vital function in constraining tumor development [14], and as such it has been postulated that many more neoplasms arise than those that eventually develop to fully malignant disease. Epidemiologic data support this contention, as evidenced by the increased incidence of viral-associated cancers [15], including human papillomavirus-related cervical and squamous carcinoma, herpesvirus-8-associated Kaposi's sarcoma and Epstein–Barr virus-related non-Hodgkin's lymphoma in immunocompromised individuals [15-19]. Similar to viral-associated cancers, there are data revealing an increased incidence of carcinogen-associated cancers in immune-compromised populations, including melanoma and lung adenocarcinoma [17,20]. Where carcinogen exposure and pathogen exposure are not thought to be etiologic factors, however, immune-compromised women exhibit reduced relative risk for common epithelial cancers, including breast adenocarcinoma [17,20-23]. Together, these epidemiological studies indicate that the overall risk for, and development of, (breast) cancer may, in part, be regulated by the immune status of the individual.

Adaptive immunity and carcinoma development: a role for B lymphocytes

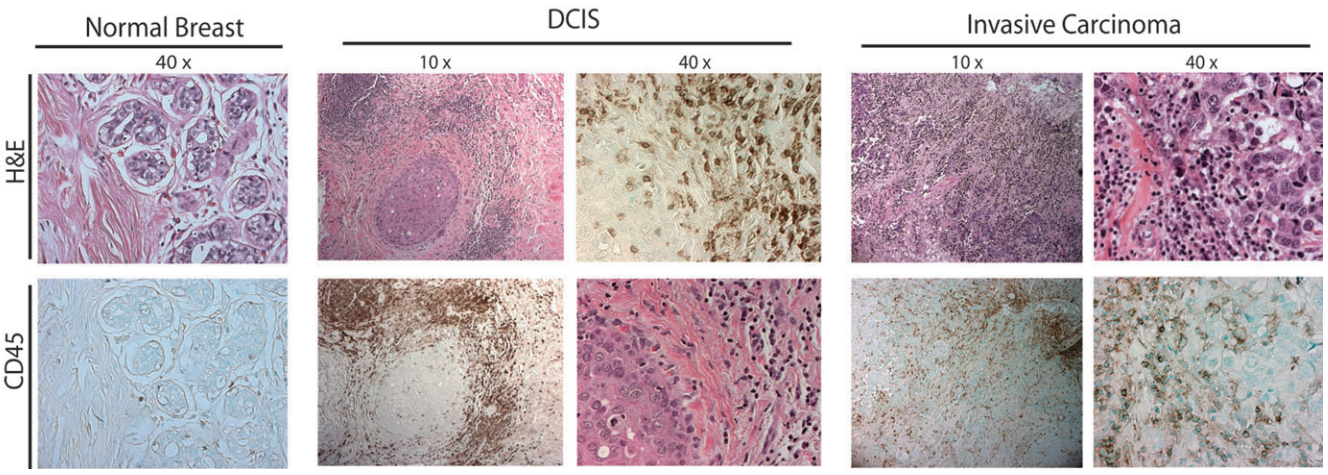
A growing body of literature has emerged demonstrating that leukocytes functionally contribute to the development of most human solid tumors. Leukocytic infiltrates into the neoplastic stroma increase, paralleling breast tumorigenesis (Figures 1 and 2). Profiles of these immune cells vary but, in general, include both lymphoid and myeloid lineages.

B lymphocytes are recognized as contributing to anticancer immune responses via their secretion of antigen-specific immunoglobulins. While it is clear that acute activation of B cells may play a role in eradicating early neoplastic cells, or may participate in spontaneous regression of tumors through classical and well-studied immunoglobulin-mediated mechanisms, recent data also indicate that chronic activation of B cells may paradoxically play a role in potentiating carcinoma development (Figure 3).

B-cell precursors mature within bone marrow, where somatic recombination of immunoglobulin genes results in expression of a diverse array of B-cell receptors. Mature antigen-committed B cells migrate to secondary lymphoid organs (lymph nodes or spleen, predominantly). Upon antigen recognition by B-cell receptors, the B lymphocytes become activated and undergo clonal expansion, resulting in their enhanced capacity to recognize foreign antigens [24]. Acute activation of B-lymphocyte responses (to foreign antigens or tissue damage) can also result in rapid induction of several soluble mediators, including diverse immunoglobulin subtypes, B-cell-derived cytokines such as IL-6 and activation of complement cascades, which together trigger recruitment of innate immune cells from the circulation. In this manner, acutely activated B cells orchestrate phagocytic or cytotoxic destruction of immunoglobulin-complexed antigens (pathogens or damaged cells) by innate immune cells. Such acute B-cell responses are critical for protecting tissues from pathogens and nonself-antigens. Chronic activation of B cells can be deleterious, however, as evidenced by their association with several pathologic disease states (rheumatoid arthritis and other autoimmune diseases) and some cancer types [25].

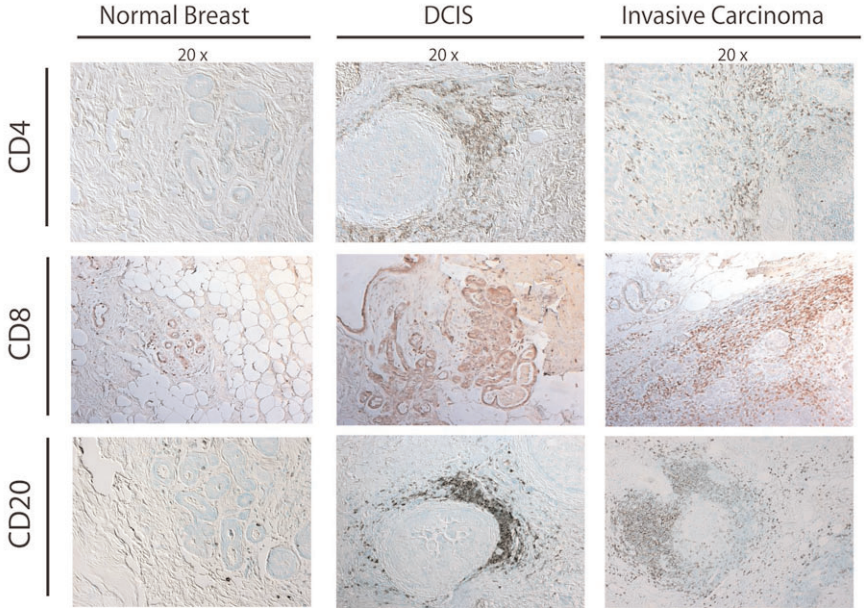
During breast carcinogenesis, mature B cells (including naive cells and activated cells) can be found in secondary lymphoid tissues as well as in tumor-associated stroma (Figure 2). As compared with healthy patients without evidence of cancer, the sentinel (draining) lymph nodes of breast cancer patients contain enriched populations of proliferating and affinity matured (IgG^+) B lymphocytes [26]. Moreover, data from retrospective studies examining the percentages of B cells present in sentinel and auxiliary lymph nodes of breast cancer patients reveal that their presence and/or maturation (IgG^+) correlates with increases in disease stage (stage I versus stage II) and in total tumor burden [27,28]. Urdiales-Viedma and colleagues utilized immunohistochemical detection of IgA, IgG and IgM in axillary lymph nodes from 50 unselected

Figure 1



Development of human breast carcinoma is characterized by abundant infiltration of immune cells. Representative sections of normal, premalignant, and malignant human breast tissue stained with hematoxylin and eosin (H&E) (upper panels), and following immunodetection of CD45 (leukocyte common antigen, brown staining). DCIS, ductal carcinoma *in situ*.

Figure 2

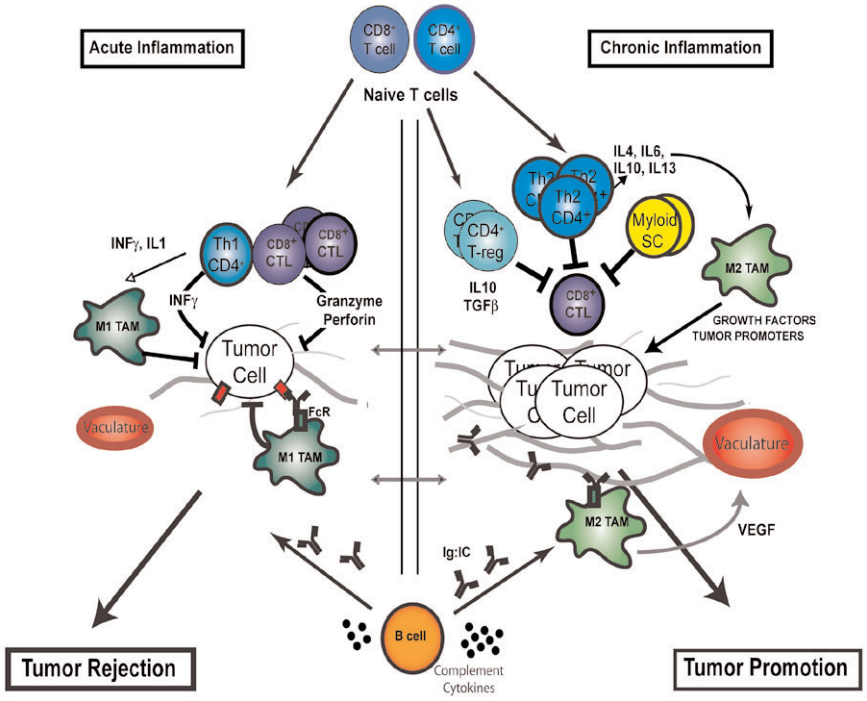


Development of human breast carcinoma is characterized by abundant infiltration of lymphocytes into neoplastic tissue. Representative sections of normal, premalignant, and malignant breast assessed for the presence of $CD4^+$ (upper panels) and $CD8^+$ (middle panels) T lymphocytes (brown staining in panels), as well as $CD20^+$ B lymphocytes (red staining in lower panels), demonstrating the extent of immune-cell infiltration into premalignant and malignant stroma. DCIS, ductal carcinoma *in situ*.

ductal breast carcinomas, and found that lymph nodes with IgG^+ lymphoid follicles and/or metastatic lymph nodes with IgM^+ lymphoid cells were statistically related to breast tumors of high histologic grade and more than three lymph node metastases [29].

Similarly, B cells present in breast tumor-associated stroma may also play a role in disease progression. Several studies have reported that infiltrating B lymphocytes represent the predominant lymphocytic population (in excess of T lymphocytes) in premalignant breast tissue, including hyperplasia

Figure 3



Contrasting roles of adaptive leukocytes during cancer development. During acute inflammatory responses (left panel), Th1 $CD4^+$ and $CD8^+$ T cells directly regulate tumor cell cytotoxicity, while indirectly polarizing innate immune cells toward tumor suppression (such as M1 polarization of tumor-associated macrophages [TAMs]). B-cell-derived factors (immunoglobulins and complement) facilitate recruitment of innate leukocytes and targeted destruction of neoplastic cells. During chronic inflammation, however (right panel), myeloid suppressor cells, Th2 $CD4^+$ T cells and regulatory T (T-reg) cells function in combination to both repress $CD8^+$ cytotoxicity and to induce protumoral polarization of innate immune response (such as M2 polarization of TAMs) via cytokine secretion (IL-4, IL-13, IL-10, IL-6 and transforming growth factor beta (TGFβ)). Chronically activated B cells promote accumulation of innate cells in the neoplastic stroma by immunoglobulin and cytokine production. When polarized, as during chronic inflammation, these innate immune cells in turn provide a rich proangiogenic and protumoral microenvironment. CTL, cytotoxic T lymphocyte; FcR, Fc receptor; INF, interferon; SC, suppressor cells; VEGF, vascular endothelial growth factor.

and early ductal carcinoma *in situ* (DCIS) [30,31] (Figure 2). Approximately 20% of invasive breast cancers contain high numbers of B cells; when present, these cells can comprise up to 60% of the neoplasia-associated lymphocyte population [32]. These observations are not specific to breast cancer – 70% of solid tumors contain elevated populations of B cells [33]. Although the presence of chronically activated B cells in breast cancer patients is clear, how these cells might be effecting disease progression has not been established. In DCIS and invasive carcinoma, tumor-associated B cells are typically found in perivascular locales clustering in aggregates with T cells, forming ectopic follicles [32,34,35]. These follicles contain B cells interdigitated around $CD21^+$ follicular DCs, thus identifying them as authentic ectopic follicles. Formation of ectopic follicles containing mature plasma cells indicates that the presence of B cells in neoplastic mammary tissue is the result of chronic activation rather than nonspecific chemoattraction. Extra-nodal B-cell proliferation and ectopic follicle formation have also been described in several autoimmune diseases (rheumatoid arthritis, multiple sclerosis, Sjogren's disease and Graves disease), where they are thought to underlie the disease pathogenesis [36-39].

How might B lymphocytes regulate carcinoma development? A vast literature exists describing the occurrence of (auto)-antibodies in either the serum of cancer patients or in interstitial antibody deposition in tumors [40]. An early presence of autoantibodies (in particular, antinuclear antibodies and smooth muscle antibodies) in serum of cancer patients is well known to correlate with an unfavorable prognosis [41]. Approximately 50% of breast cancer patients contain circulating immunoglobulins that specifically react with tumor-derived antigens – autoantibodies against ErbB2/HER2/neu are present in 20% of patients with ErbB2-positive breast cancer, making it the most common breast cancer 'autoantigen' [42]. Paradoxically, the presence of specific autoantibodies in serum and/or at tumor sites correlates with poor patient survival [40,41,43] – perhaps indicating that immunoglobulins resulting from chronic B-cell activation in response to tumor-specific antigens might promote disease progression (Figure 3).

Despite the presence of antitumor antibodies in greater than one-half of all breast cancer patients, there are only few reports of spontaneous tumor regression (presumed to be immunologic) in the absence of therapy [44,45]. Several factors may influence the efficiency of antitumor antibodies in inducing tumor regression/destruction, including the immunoglobulin concentration, HLA expression, tumor tolerance/immune suppression, and impaired cytotoxic T-cell activity. Whether individuals with progressing tumors harbor a higher antigen load that thus triggers enhanced immunoglobulin production, or whether an increased presence of serum or interstitial immunoglobulins predisposes patients to development of more advanced or recurrent cancers, therefore requires further study. While the answer is not clear, the combined implication of the data is that B lymphocytes do play a role in human carcinoma development, therefore necessitating a mechanistic evaluation of their role and specificity to determine whether they represent tractable targets for anticancer therapy.

Adaptive immunity and carcinoma development: a role for T lymphocytes

Histochemical detection of T lymphocytes in archival human carcinoma tissues has revealed that they also are commonly associated with developing carcinomas (Figure 2); however, the prognostic significance of T-cell infiltrations during breast carcinogenesis is subject to great debate. While B cells appear to represent the predominant lymphocyte during early breast cancer progression [34], infiltrating T lymphocytes (both $CD4^+$ and $CD8^+$) are more extensive in higher grade DCIS and invasive carcinomas [46].

The extent of T-cell infiltration into invasive breast carcinomas has been reported to range from 1% to 45% of the total cellular mass [47]. In rapidly proliferating tumors, the presence of T lymphocytes (by histopathological determination) at tumor sites is a good prognostic indicator when compared with nonimmunogenic tumors, and correlates with auxillary lymph node negativity, a smaller tumor diameter, a lower histological grade and recurrence-free survival [48] – therefore supporting an overall role for T cells in immune surveillance. The exact composition of T lymphocyte infiltration varies greatly, however, and may profoundly affect disease progression and overall patient survival.

During breast carcinogenesis, the presence of metastatic mammary epithelial cells in sentinel lymph nodes draining the primary tumor represents the strongest prognostic indicator for disease progression and overall patient outcome [49-51]. While it is unclear whether the presence of $CD8^+$ CTLs alone provides any prognostic information, the presence of high percentages of $CD4^+$ T-helper cells at primary tumor sites positively correlates with disease progression, including metastatic spread to sentinel lymph nodes and increased primary tumor size [47,52]. Perhaps more significant is the ratio of $CD4^+$ to $CD8^+$ cells, where primary tumors with ratios greater than one correlate with lymph node metastasis and reduced patient survival [47,52]. Similar results have been reported for colorectal carcinoma [53], renal carcinoma, esophageal carcinoma, and small-cell lung carcinoma [54].

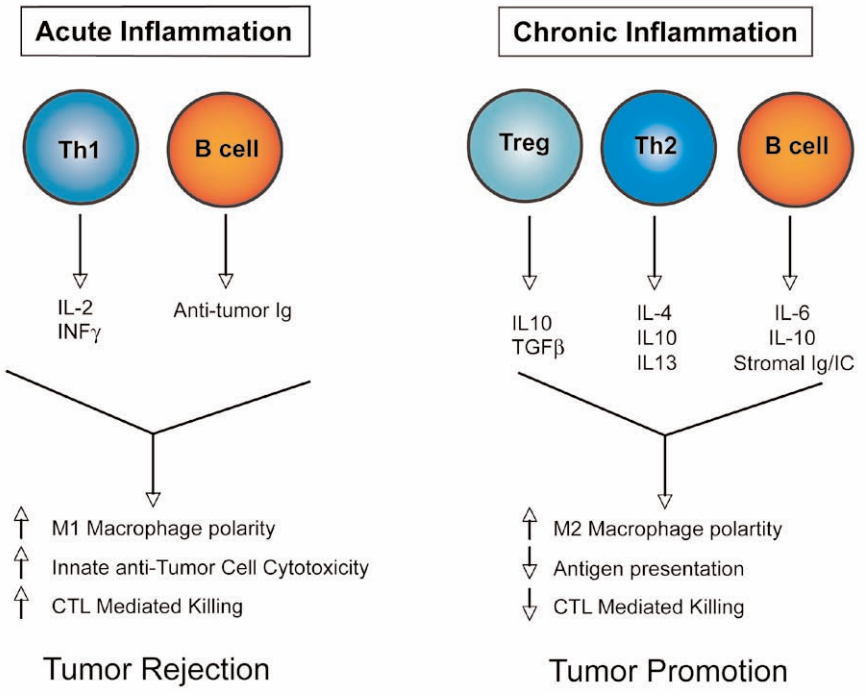
Why are $CD8^+$ CTL-mediated responses not more effective in eradicating or minimizing cancer occurrence and how might $CD4^+$ T cells be involved in enhancing breast cancer progression? One plausible mechanism may have to do with the 'polarity' of the $CD4^+$ T-helper-cell response at primary tumor sites and/or their distant metastases (Figure 3). $CD4^+$ T-helper cells are activated in response to soluble factors and can be classified generally into two categories as either Th1 cells or Th2 cells (Figure 4) [55]. Following an activating stimulus, $CD4^+$ T-helper cells that are Th1-polarized secrete IFNγ, transforming growth factor beta, TNFα and IL-2 [56]. These cytokines collaborate with the cytotoxic/cell killing functions of $CD8^+$ T cells [57] and can induce upregulation of antigen processing (in the proteasome), can induce expression of MHC class I and II molecules, and can induce other antigen display cofactors in neoplastic cells. Th1 $CD4^+$ T-helper cells also enhance antitumor immune responses by secretion of INFγ, which in turn induces activation of macrophage cytotoxic activity [58]. In contrast Th2-polarized $CD4^+$ T-helper cells express IL-4, IL-5, IL-6, IL-10 and IL-13, which induce T-cell anergy and loss of T-cell-mediated cytotoxicity while also enhancing humoral immunity (B-cell function) [59]. Taken together, Th1 responses are thought to be beneficial toward antitumor immunity [60-63] whereas Th2 responses may downregulate cell-mediated antitumor immunity [63-67] and enhance protumor humoral responses [68,69].

Role for regulatory T lymphocytes

In addition to T-helper-cell polarity, developing neoplasms can escape $CD8^+$ T-cell cytotoxicity by promoting expansion of regulatory T cells (T_{reg}). T_{reg} cells normally function to protect tissue from autoimmune disease by suppressing self-reactive cells. Typically characterized by expression of CD4, CD25 and FOXP3, T_{reg} cells can account for approximately 5–10% of all T lymphocytes in healthy tissues.

In breast cancers the percentage of T_{reg} cells, as assessed by FOXP3 positivity, increases in parallel with the disease stage, from normal to DCIS and from DCIS to invasive carcinoma [70]. In patients with invasive carcinoma the presence of high numbers of $FOXP3^+$ T cells predicts worse relapse-free survival and decreased overall patient survival [70], and may indicate that the presence of T_{reg} cells promotes tumor progression by inhibiting immunosuppression. The mechanisms underlying immunosuppression are not clear; however, $CD4^+CD25^{high}FOXP3^+$ T_{reg} cells retain the ability to actively inhibit $CD4^+CD25^-$ T cells, $CD8^+$ T cells, DCs, NK cells, and B cells in a cell-to-cell contact and dose-dependent manner [71-75]. The mechanisms by which developing tumors might stimulate proliferation and differentiation of T_{reg} lymphocytes

Figure 4



Model depicting the consequences of acute inflammation versus chronic inflammation. During acute antitumor inflammatory responses (left panel), Th1-polarized T cells secrete antitumor cytokines (IL-2 and INFγ, for example), which in combination with antitumor-directed B-cell-derived factors (such as immunoglobulins (Igs)) activate tumor inhibitory responses in recruited innate immune cells and cytotoxic T lymphocytes (CTLs) that together favor tumor rejection. In contrast, chronic activation of immune response (right panel) without resolution (of damage) often results in accumulation of regulatory T (Treg) cells, Th2 cells, and activated B cells, which in turn secrete progrowth factors (IL-4, IL-6, IL-10, IL-13, transforming growth factor beta (TGFβ) and immunoglobulins, for example) that enhance protumor responses in innate immune cells and inactivate CTL cytotoxicity, thus favoring tumor promotion.

are still not well known, but production of prostaglandin E_2 by tumor cells and of CCL22 by tumor-associated macrophages may act as chemotactic and differentiation agents for T_{reg} cells [76-79].

Acquired immunity and carcinoma development

It is well established that chronic infiltration of tissue by some innate immune cell types (for example, immature monocytes, macrophages, mast cells or neutrophils) contributes to epithelial cancer development [3,8,80]. Many studies have reported that inflammatory leukocytes promote cancer development due to their enormous plasticity and their capacity to produce a myriad of cytokines, chemokines, metalloserine and metallocysteine proteases, reactive oxygen species, histamine and other bioactive mediators [3,8,80]. Several physiological processes necessary for tumor development, such as enhanced cell survival, tissue remodeling, angiogenesis and suppression of antitumor adaptive immune responses, are thus regulated by leukocytic infiltrates in neoplastic environments. This regulation is exemplified by positive correlations between numbers of innate immune cells (macrophages, mast cells and neutrophils) infiltrating human tumors with the number of blood vessels [81,82], and by experimental findings in mouse models where attenuating innate immune cell infiltration of premalignant tissue reduces angiogenesis and limits tumor development [83-90].

The earliest reports revealing a potential tumor-enhancing effect of adaptive immunity found that passive transfer of tumor-specific antibodies enhanced *in vivo* outgrowth of transplanted tumor cells or chemically induced tumors [91-93]. More recently, the availability of de novo carcinogenesis mouse models for the study organ specific cancer development has allowed experimentalist to revisit these analysis. Their experiments have revealed that the tumor enhancing roles of humoral immunity are more involved in innate immune cell responses, as opposed to direct regulation of neoplastic cell survival. For example, active immunization of mice harboring a mutant *ras* oncogene resulted in activation of humoral immune responses and enhanced papilloma formation upon chemical promotion [94,95].

Studies by Barbera-Guillem and colleagues reported that 'antitumor' humoral immune responses potentate *in vivo*

growth and invasion of injected murine and human tumor cell lines via recruitment and activation of protumor granulocytes and macrophages [94,96,97]. Once in the neoplastic microenvironment, deposited immunoglobulin may mediate recruitment of innate immune cells via activation of complement cascades or engagement with Fc receptors expressed on resident or recruited innate immune cells that trigger various cellular responses, including phagocytosis, antigen presentation, secretion of proinflammatory mediators and antibody-dependent cytotoxicity [98,99]. During breast cancer development, immunoglobulin deposition in neoplastic stroma is known to increase the bioavailability of vascular endothelial growth factor (VEGF) by binding to Fc receptors on tumor-associated macrophages, thus triggering release of VEGF into the interstitium [96]. Moreover, the presence of endocytosed immunoglobulin in macrophages in breast carcinoma tissues corresponds with local extracellular VEGF protein levels and local angiogenic vascular buds [96].

The significance of tumor-associated macrophages has been experimentally addressed in a mouse model of mammary carcinogenesis; that is, mouse mammary tumor virus–polyomavirus middle T antigen transgenic mice [86]. In this model of mammary carcinogenesis, infiltration of macrophages into premalignant mammary tissue is associated with tumor progression [100]. Failure to recruit macrophages into neoplastic tissue does not alter the hallmarks of premalignancy, but instead significantly delays development of invasive carcinomas and results in reduced pulmonary metastases [86]. Metastatic potential is restored by transgenic expression of colony-stimulating factor (CSF)-1 in the mammary epithelium of CSF-1-deficient/polyomavirus middle T antigen mice [86]. Macrophage stimulation of mammary tumor progression is at least in part due to their ability to enhance angiogenesis via regulating VEGF levels and bioavailability [101]. These experimental data combined with the positive correlation between CSF-1 levels, macrophage recruitment and poor prognosis in human cancers [102] support the notion that macrophages and/or their products play a major role in facilitating late-stage metastatic progression of tumors [86]. Other cells of myeloid lineage have also been reported to contribute to tumor development – NK cells can play a role in protection against experimental tumor growth, in part by producing mediators with antiangiogenic properties [103,104]. These studies have together induced a paradigm shift regarding the role of immune cells during malignant progression. Whereas the historical viewpoint suggested that host immunity is protective with regards to cancer, it is now clear that certain subsets of chronically activated innate immune cells promote growth and/or facilitate survival of neoplastic cells.

In addition to providing a survival advantage to evolving neoplastic cells through their release of protumor mediators, is there evidence that recruited innate immune cells regulate any antitumor bioactivities of T cells present in premalignant/malignant mammary tissue? Neoplastic microenvironments favor polarized chronic protumorigenic inflammatory states, as opposed to those representing acute antitumor immune responses [8,105]. Clinical data indicate that the 'immune status' of healthy individuals is distinct as compared with those harboring malignant tumors, where T lymphocytes in the later population are found to be functionally impaired [106]. A subset of innate immune cells (for example, myeloid suppressor $CD11b^+Gr\text{-}1^+$ cells) accumulate in the peripheral blood of cancer patients [107,108], as well as in tumors and lymphoid organs [105,108,109]. Myeloid suppressor cells are known to induce T-lymphocyte dysfunction by direct cell–cell contact and by production of immunosuppressive mediators, and thus actively inhibit antitumor adaptive immunity [108,109]. Myeloid suppressor cells can also directly promote tumor growth by contributing to tumor-associated angiogenesis [110]. In addition, malignant lesions attract regulatory T cells that can suppress effector functions of CTLs [105]. Immune states such as these disable tumor-killing $CD8^+$ CTL responses and enable states of immune privilege that foster escape from antitumor immunity while simultaneously exploiting activated innate immune cells that enhance cancer development. The degree to which similar mechanisms are at play during breast carcinogenesis is currently being investigated.

Cytokines derived from activated humoral immunity and/or Th2 T lymphocytes also actively regulate chemoattraction and polarization of tumor-associated leukocytes, especially macrophages. Chronically activated B cells (typically in germinal centers or in ectopic follicles) can produce granulocyte–macrophage-CSF, TNFα, IL-6, and IL-10 [111]. These cytokines, in combination with Th2 cytokines such as IL-4, IL-13, and IL-10, are potent effectors of innate immune cell polarization. Protumor M2 polarization in macrophages is induced by the Th2/humoral cytokines IL-4 and IL-10, while simultaneously repressing antitumor M1 macrophage polarization [112]. Granulocyte–macrophage-CSF, IL-6 and IL-10 secreted by activated B cells suppress macrophage cytotoxic activity [112], while IL-10 inhibits both antigen presentation by macrophages as well as monocyte differentiation into DC lineages [113]. Taken together, factors derived from chronically activated lymphocytes sculpt innate immune cell responses towards tumor tolerance and promotion of disease progression.

Conclusions

During the past decade, insights have been gained regarding mechanisms underlying the dynamic interplay between immune cells and tumor progression. The accumulated data indicate that the outcome of an immune response toward a tumor is largely determined by the type of immune response elicited (Figure 3). A tumor-directed immune response involving cytolytic $CD8^+$ T cells, Th1 cells and NK cells appears to protect against tumor development and progression. If, on the other hand, the immune response involves B cells and

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activation of humoral immunity, and/or a Th2 polarized response the probable outcome is promotion of tumor development and progression. This balance between a protective cytotoxic response and a harmful humoral or Th2 response can be regulated systemically by the general immune status of the individual, as well as locally by myeloid suppressor cells and T_{reg} cells, and thus offers clinicians attractive targets for anticancer immune-based therapies.

Competing interests

The authors declare that they have no competing interests.

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Research Article

Distinctive Features of Angiogenesis and Lymphangiogenesis Determine Their Functionality during *De novo* Tumor Development

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Abstract

Blood and lymphatic vasculature are essential components of all organs, responsible for maintaining organ fluid dynamics and tissue homeostasis. Although both vessel systems are composed of similar lineages of endothelial cells whose crude functions include fluid and cell transport, each system also possesses distinctive physiologic properties, enabling their distinctive functions in tissues. The role of hematogenous vasculature and development of angiogenic blood vessels during cancer development is well established; however, the role of lymphangiogenesis and structural/functional alterations occurring within lymphatic vessels during cancer development are incompletely understood. To assess premalignant versus malignant alterations in blood and lymphatic vasculature associated with squamous epithelial skin carcinogenesis, we assessed architectural and functional features of both vascular systems using a mouse model of *de novo* carcinoma development. We report that, as vasculature acquires angiogenic and/or lymphangiogenic properties, angiogenic blood vessels become leaky in premalignant tissue and at peripheries of carcinomas, where enlarged lymphatic capillaries efficiently drain increased tissue fluid, thereby maintaining tissue hemodynamics. In contrast, central regions of carcinomas exhibit elevated tissue fluid levels, compressed lymphatic lumina, and decreased vascular leakage, thus indicating impaired hemodynamics within solid tumors. Together, these data support the notion that therapeutic delivery of anticancer agents is best realized in premalignant tissues and/or at the peripheries of solid tumors where hemodynamic forces support drug delivery. Strategies to normalize intratumoral hemodynamics would therefore enhance therapeutic delivery to otherwise poorly accessible central regions of solid tumors. [Cancer Res 2007;67(11):5211–20]

Introduction

Blood and lymphatic vessels comprise two interdependent vascular networks in all tissues. Whereas blood vessels deliver blood cells, plasma proteins, and oxygen to tissues, lymphatics, composed of lymph-forming capillaries and collecting vessels, continually regulate interstitial fluid pressure by draining interstitial fluid and debris to maintain tissue homeostasis. When tissues are acutely damaged, activation of both vascular systems occurs as part of innate repair programs; once complete, both systems return to their homeostatic states. In contrast, sustained activation of one or both vascular networks is associated with some chronic disorders, such as rheumatoid arthritis (1) and psoriasis (2, 3), and contributes to disease pathogenesis. Cancer development is similarly associated with chronic activation of blood vasculature (i.e., angiogenesis) in premalignant and malignant tissues (4). Activation of angiogenic vasculature in premalignant tissue is characterized by increased proliferation of vascular endothelial cells (VEC) and sprouting of new immature leaky blood vessels from preexisting vascular beds (5). Increased leakage of plasma proteins from immature angiogenic vessels leads to increased interstitial fluid content, lymph formation, and drainage via lymphatic vessels back into the blood circulation (6). High interstitial fluid pressure (IFP), which forms a barrier to transcapillary transport, results in inefficient delivery of therapeutic drugs from vasculature into tumor stroma (7). It has been postulated that high IFP, common to many solid tumors, is in part a result of inefficient clearance of tissue fluid by lymphatic vessels (7). This postulate is supported by histochemical studies evaluating lymphatic architecture and diminished lumen diameters in archival human carcinomas and murine xenograft tumors (8–12). Studies evaluating functional changes in the status of lymphatic endothelial cells (LEC) and/or lymphatic vessels compared with VECs and blood vessels in premalignant tissues have not been well described.

To critically examine distinctive versus common physiologic and functional properties of blood versus lymphatic vasculature that accompany and/or contribute to cancer development, we used a transgenic mouse model of *de novo* epithelial squamous cell carcinogenesis (e.g., K14-HPV16 mice; ref. 13). HPV16 mice progress through well-defined premalignant stages before *de novo* carcinoma development, mirroring histopathologic stages observed during human cervical carcinogenesis (14). HPV16 mice develop hyperplastic skin lesions with 100% penetrance by 1 month of age that focally progress to dysplasia by 3 to 6 months (13). Precursor dysplasias undergo malignant conversion into varying grades of squamous cell carcinoma (SCC) in skin in 50% of mice that metastasize to regional lymph nodes with a 30% frequency (13, 15). Angiogenic vasculature is first evident in premalignant hyperplasias, development of which is linked to infiltration of innate immune cells (e.g., mast cells, granulocytes, and macrophages; refs. 15–18). Using this model, we assessed molecular, histopathologic, and functional variables of blood and lymphatic vasculature to reveal their distinctive physiologic properties at each stage of neoplastic development.

Materials and Methods

Animal Husbandry, Genotype, and Histopathologic Analyses

All animals were maintained within the University of California at San Francisco (UCSF) Laboratory for Animal Care barrier facility according to

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Institutional Animal Care and Use Committee procedures. HPV16 transgenic mice (19), preparation of tissue sections (ear skin) for histologic examination, and characterization of neoplastic stages based on H&E histopathology and cytokeratin intermediate filament expression have been described previously (13, 15, 20). Paraffin-embedded tissue sections were fixed by immersion in 10% neutral-buffered formalin, dehydrated through graded ethanol and xylenes, embedded in paraffin, cut with a Leica 2135 microtome into 5-μm-thick sections, and histopathologically examined following H&E staining and immunoreactivity of keratin intermediate filaments. Hyperplastic lesions were identified by a 2-fold increase in epidermal thickness and an intact granular cell layer with keratohyalin granules. Dysplastic lesions were characterized based on basal and spinous cell layers with hyperchromatic nuclei representing greater than half of the total epidermal thickness and incomplete terminal differentiation of keratinocytes. SCC was identified by abundance of abnormal mitotic figures and an invasive loss of integrity in epithelial basement membrane with clear development of malignant cell clusters proliferating in the dermis. SCCs were graded as has been described previously (17).

Flow Cytometry

Single cell suspensions were prepared from ear ($n = 4$) and tumor ($n = 7$) tissue as described previously (21). Cells were incubated for 10 min at 4°C with rat anti-mouse CD16/CD32 monoclonal antibody (mAb; BD Biosciences) at a 1:200 dilution in PBS/bovine serum albumin (BSA) to prevent nonspecific antibody binding. Subsequently, cells were washed and incubated for 20 min with phycoerythrin-conjugated anti-mouse CD31 (1:200; eBioscience) and anti-mouse podoplanin hybridoma supernatant (mAb clone 8.1.1, 1:1000; Developmental Studies Hybridoma Bank, University of Iowa). Cells were washed twice with PBS/BSA and incubated with Alexa Fluor 647–conjugated anti-hamster antibody (1:1,000; Molecular Probes) for 20 min. After two washes with PBS/BSA, 7-aminoactinomycin D (1:10; BD Biosciences) was added to discriminate between viable and dead cells. Data acquisition was done on a FACSCalibur using CellQuestPro software (BD Biosciences), and data analysis was done using FlowJo software (Tree Star, Inc.). Data shown represent the mean ± SE. P values of <0.05 were considered to be statistically significant.

Immunohistochemistry

Immunohistochemistry on paraffin-embedded tissue sections. Tissue sections were deparaffinized, briefly washed in PBS, and blocked for 15 min in blocking buffer (5.0% normal goat serum/2.5% BSA/PBS). Primary antibodies were diluted in 0.5× blocking buffer: rat anti-mouse CD31 (1:50; PharMingen), Syrian hamster anti-mouse podoplanin (1:200, clone 8.1.1; Developmental Studies Hybridoma Bank, University of Iowa), guinea pig anti-mouse cytokeratin pan (1:100; Progen Biotechnik GmbH), and rabbit anti-mouse lymphatic vessel endothelial receptor-1 (LYVE-1; 1:200; Upstate). Sections were incubated with primary antibody overnight at 4°C in a humidified chamber followed by three brief washes in PBS and subsequently incubated with secondary antibodies Alexa Fluor 488–conjugated anti-rat antibody (1:500; Molecular Probes), Alexa Fluor 633–conjugated anti-guinea pig antibody (1:500; Molecular Probes), Alexa Fluor 594–conjugated anti-hamster antibody (1:500; Molecular Probes), or Alexa Fluor 594/488–conjugated anti-rabbit antibody (1:500; Molecular Probes) for 2.0 h at room temperature in a humidified chamber. After five 3-min washes in PBS, the fluorescently stained sections were mounted in ProLong Gold mounting medium (Molecular Probes). Fluorescence was visualized using a laser scanning confocal microscope LSM510 META (Carl Zeiss MicroImaging, Inc.) and analyzed with Zeiss LSM Image Examiner. Ear tissue ($n = 4$/time point) and tumor tissue ($n = 5$) were assessed for lymphatic luminal status and blood and lymphatic vessel density by counting the number of vessels in five random high-power fields of vision per time point per tissue, tumor center, and periphery.

Immunohistochemical detection of proliferating endothelial cells. Fresh bromodeoxyuridine (BrdUrd; Sigma) suspended in water (1.0 mg/mL) was given to mice in drinking water *ad libitum* and changed every 2nd day for a total of 8 days. For fluorescent angiography, animals were tail vein injected with FITC-conjugated *Lycopersicon esculentum* (tomato) lectin (2.0 mg/mL; Vector Laboratories) 3.0 min before cardiac perfusion with the following reagents in chronological order: 15 mL of 1% paraformaldehyde/0.5% glutaraldehyde, 7.0 mL PBS, 15 mL PBS containing 1.0% BSA, and 15 mL of 4.0% paraformaldehyde at a constant flow rate of 7.0 mL/min. Tissue was harvested and fixed in 10% zinc-buffered formalin for 8 to 16 h at 4°C before further processing followed by dehydration through graded alcohols and xylene and embedding in paraffin. Paraffin sections (5 μm thick) were cut using a Leica 2135 microtome. Blood vasculature was detected by fluorescent angiography (22), and immunohistochemical analysis was done detecting lymphatic vessels (LYVE-1 reactivity), keratin-expressing epithelial cells (pan-keratin reactivity), proliferating nuclei (BrdUrd incorporation), and all nuclei as follows: Sections were deparaffinized, incubated in 2.0 N HCl for 1.0 h at room temperature, rinsed in PBS, and blocked for 30 min in blocking buffer (5.0% normal goat serum/2.5% BSA/PBS). Primary antibodies were diluted in 0.5× blocking buffer: guinea pig anti-mouse cytokeratin pan (1:100), biotinylated anti-BrdUrd (1:50; clone Br-3; Caltag), and rabbit anti-mouse LYVE-1 (1:200). Sections were incubated with primary antibody overnight at 4°C followed by three brief washes in PBS and subsequently incubated with secondary antibodies (all 1:500; Molecular Probes) Alexa Fluor 633–conjugated anti-guinea pig antibody, Alexa Fluor 546-streptavidin–conjugated antibody, and Alexa Fluor 594–conjugated anti-rabbit antibody for 2.0 h at room temperature in a humidified chamber. After five 3-min washes in PBS, the fluorescently stained sections were subjected to a nuclear staining with SYTO62 (0.7 μmol/L in H_2O; Molecular Probes) for 10 min and then mounted in ProLong Gold mounting medium. Fluorescence was visualized using a laser scanning confocal microscope LSM510 META and analyzed with a Zeiss LSM Image Examiner. A minimum of 100 endothelial cell nuclei in control ($n = 4$), premalignant ear ($n = 4$), and tumor tissue ($n = 7$) was identified in tissue sections based on their morphologic protrusion into the vessel lumen, and the percentage of proliferating nuclei was determined.

Fluorescent Angiography and Immunohistochemistry of Whole-Mount Ear Tissue and Thick Frozen Tumor Sections

Whole-mounted ear tissue ($n = 3$ per time point) was prepared as described previously (23). Briefly, mice were anesthetized with a 2% isofluorane/98% oxygen mixture and 100 μL of FITC-conjugated *L. esculentum* (tomato) lectin (2.0 mg/mL) were injected into the tail vein and allowed to circulate for 3.0 min followed by cardiac perfusion with 35 mL of PBS-buffered 4% paraformaldehyde (pH 7.4; ref. 22). Ears were harvested, ventral and dorsal aspects were separated, and cartilage was removed from ventral aspects, which were then immersion fixed in PBS-buffered 4% paraformaldehyde (pH 7.4) overnight at 4°C and subjected to the following staining procedure under exclusion of light: Ventral aspects of ears were rinsed briefly in PBS containing 0.3% Triton X-100 (PBS-T) and blocked in PBS-T containing 3% goat serum overnight at 4°C. Tissue was subsequently incubated with primary rabbit anti-mouse LYVE-1 antibody (1:2,000; Upstate) diluted in PBS-T over two nights at 4°C. After five washes in PBS-T (5–10 min each) and a 2- to 3-h blocking incubation with PBS-T containing 3% goat serum at 4°C, tissue was incubated overnight at 4°C with a Alexa Fluor 594–conjugated anti-rabbit antibody (1:500; Molecular Probes). Tissue was then washed 10 times for 5 min at 4°C and mounted in ProLong Gold mounting medium.

Tumors ($n = 5$) were dissected, flash frozen in OCT compound, and cut into 200 μm sections and subsequently rinsed briefly in PBS-T and blocked in PBS-T containing 3% goat serum for 2.0 h at room temperature. Tissue was subsequently incubated with primary rabbit anti-mouse LYVE-1 antibody (1:2,000) diluted in PBS-T overnight at 4°C. After three 3-min washes in PBS-T, tissue was incubated for 3 h at room temperature with Alexa Fluor 594–conjugated anti-rabbit antibody (1:500). After three 3-min washes in PBS-T, tissue was mounted in ProLong Gold mounting medium. Fluorescence was visualized using a laser scanning confocal microscope LSM510 META and analyzed with a Zeiss LSM Image Examiner (Carl Zeiss MicroImaging).

Intravital Perfusion of Lymphatics

Intravital perfusion of lymphatics was modified from what has been described by Nagy et al. (24). Mice were anesthetized with a 2% isofluorane/98% oxygen mixture and cradled in a transparent acrylic resin mold

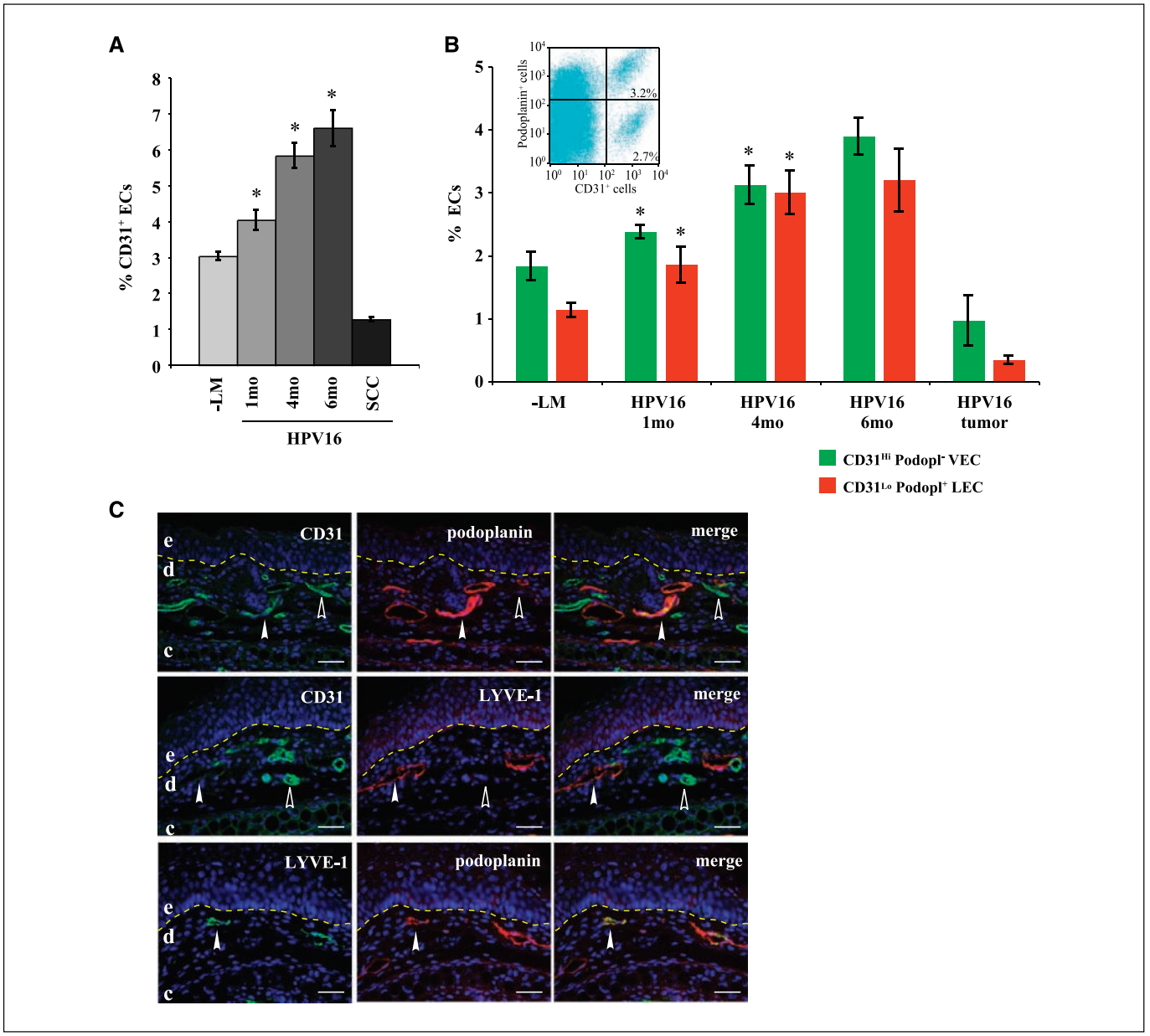

Figure 1. Increased endothelial cell content in neoplastic tissue. *A,* flow cytometric analysis of normal, premalignant, and carcinoma tissue detecting the pan-endothelial cell (*EC*) marker CD31. Percentage $CD31^+$ endothelial cells out of the total number of cells analyzed. *B,* distinction of VECs from LECs by flow cytometry based on CD31 and podoplanin expression. *Inset,* example plot from dysplastic (4 mo old) HPV16 ear tissue biopsy. *C,* immunohistochemical detection of CD31 and lymphatic-specific markers podoplanin and LYVE-1 distinguishes between $CD31^{Hi}$/podoplanin$^-$/LYVE-1$^-$ blood vessels (*open arrow*) and $CD31^{Lo}$/podoplanin$^+$/LYVE-1$^+$ lymphatic vessels (*solid arrow*). *e,* epidermis; *d,* dermis; *c,* cartilage. *Dashed line,* basement membrane. *Bar,* 50 μm. *, $P \leq 0.05$, two-tailed unpaired nonparametric Mann-Whitney *U* test.

(Syndicate Sales, Inc.). Ears (n = 6 per time point) were mounted flat on a resin support, held in place by silicone vacuum grease, and viewed in a dissecting microscope (Olympus). Colloidal carbon (Higgins non-waterproof black drawing ink; Sanford) was diluted 1:1 in PBS, filtered through a 0.22-μm filter, and injected i.d. through a prepulled borosilicate glass micropipette with an inner diameter of 1.0 μm (World Precision Instruments) attached to a 100 μL Hamilton syringe (World Precision Instruments). The micropipette was repeatedly injected into the dermis of the peripheral ear until a lymphatic vessel was entered. Colloidal carbon was then slowly injected. For lymphatic vessel diameter analysis, ear tissue (n = 3 per time point) was subsequently harvested and fixed overnight in acetone at 4°C. After fixation, the tissue was cleared in toluene 48 h at room temperature and mounted in Permount (Fisher Scientific). For photography, a digital camera (Nikon Coolpix 950) was used and the images were analyzed using Openlab software (Improvision). For *in vivo* analysis of colloidal carbon clearance, images were captured using a LUMAR microscope (Carl Zeiss MicroImaging) within 1.0 min of injection and again after 20 min. Both ears of negative littermate (−LM) and 4-month HPV16 mice (n = 3 each) were analyzed.

Miles Assay

Mice were anesthetized with a 2% isofluorane/98% oxygen mixture, and Evans blue dye (30 mg/kg in 100 μL PBS; Sigma-Aldrich) was injected into the tail vein. In some experiments, after 1 min, 30 μL of 5% mustard oil (phenyl isothiocyanate, 98%; Sigma-Aldrich) diluted in mineral oil (Sigma-Aldrich) or mineral oil as control were applied to the dorsal and ventral

surfaces of the ear; the application process was repeated 15 min later. Evans blue dye was allowed to circulate for 30 min. Anesthetized mice were subsequently cardiac perfused with 1% paraformaldehyde in 0.05 mol/L citrate buffer (pH 3.5). Ears (n = 6 per time point; n = 3 per mineral oil/mustard oil treatment) were removed, blotted dry, and weighed. Tumors (n = 4) were dissected and cut into seven pieces representing six peripheral sections and center. Premalignant nontumor tissue was derived from neck, chest, and abdomen of non–tumor-bearing HPV16 animals. Evans blue dye was extracted from ears, nontumor tissue, and tumor pieces in 1.0 mL formamide for 48 h at 60°C and measured spectrophotometrically at 610 nm in a SpectraMax 340 (Molecular Devices). Data are expressed as mean ± SE. P values of <0.05 were considered to be statistically significant.

Tissue Fluid Determination

Mice were euthanized, and ears (n = 6 per time point) and/or tumors (n = 4) were removed and weighed (wet weight). Tumors were dissected into six peripheral sections and tumor centers. Premalignant nontumor tissue was derived from neck, chest, and abdomen of non–tumor-bearing HPV16 animals. Tissue was snap frozen, lyophilized, and reweighed (dry weight). The difference between wet and dry weight reflects fluid tissue component, although dry weight reflects solid tissue component. Data are expressed as mean ± SE. P values of <0.05 were considered to be statistically significant.

In vivo High Molecular Dextran Injection

Functionality of initial lymphatics was measured as uptake and drainage of interstitial fluid containing high molecular weight (2,000,000 Da) dextran

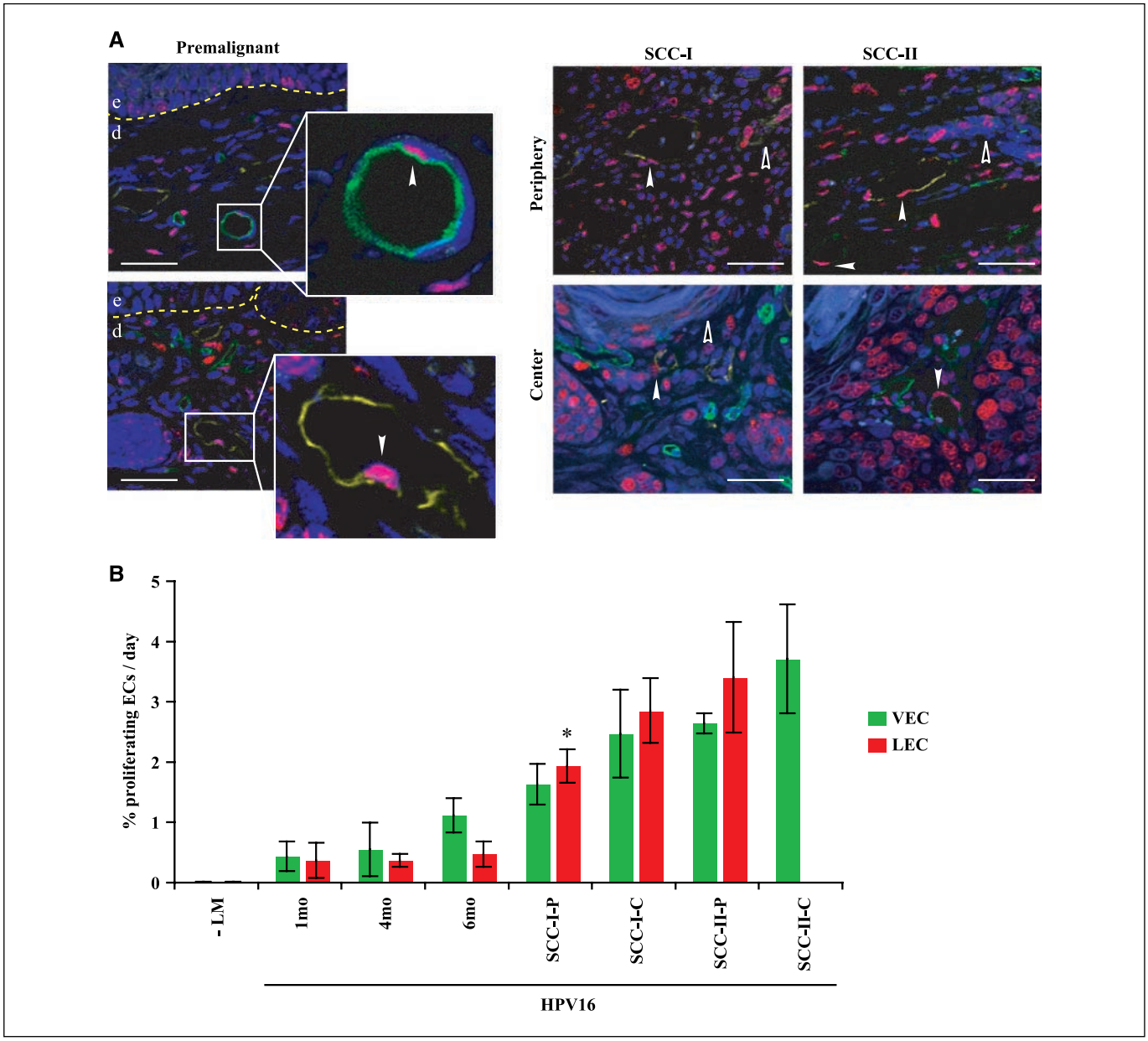

Figure 2. VEC and LEC proliferation in premalignant and carcinoma tissue. *A,* quiescent and proliferating (*red staining*) endothelial cell nuclei (*solid arrows*) in blood (*green staining*) and lymphatic (*yellow staining*) vessels in premalignant and malignant (*white staining* for cytokeratin-positive keratinocytes; *open arrow*) tissue. *Bar,* 50 μm. *e,* epidermis; *d,* dermis. *B,* quantitative analysis of proliferating VECs and LECs in −LM, premalignant, and carcinoma tissue. Proliferating LECs were identified in the periphery and center of well-differentiated grade 1 SCCs (*SCC-I*) but limited to periphery of less-differentiated grade 2 SCCs. Absence of open lumen lymphatic vessels SCC-II centers precluded analysis of LECs in that locale. *, $P \leq 0.05$, two-tailed unpaired nonparametric Mann-Whitney U test. *Dashed line,* basement membrane. *Blue staining,* SYTO62-nuclear counterstain.

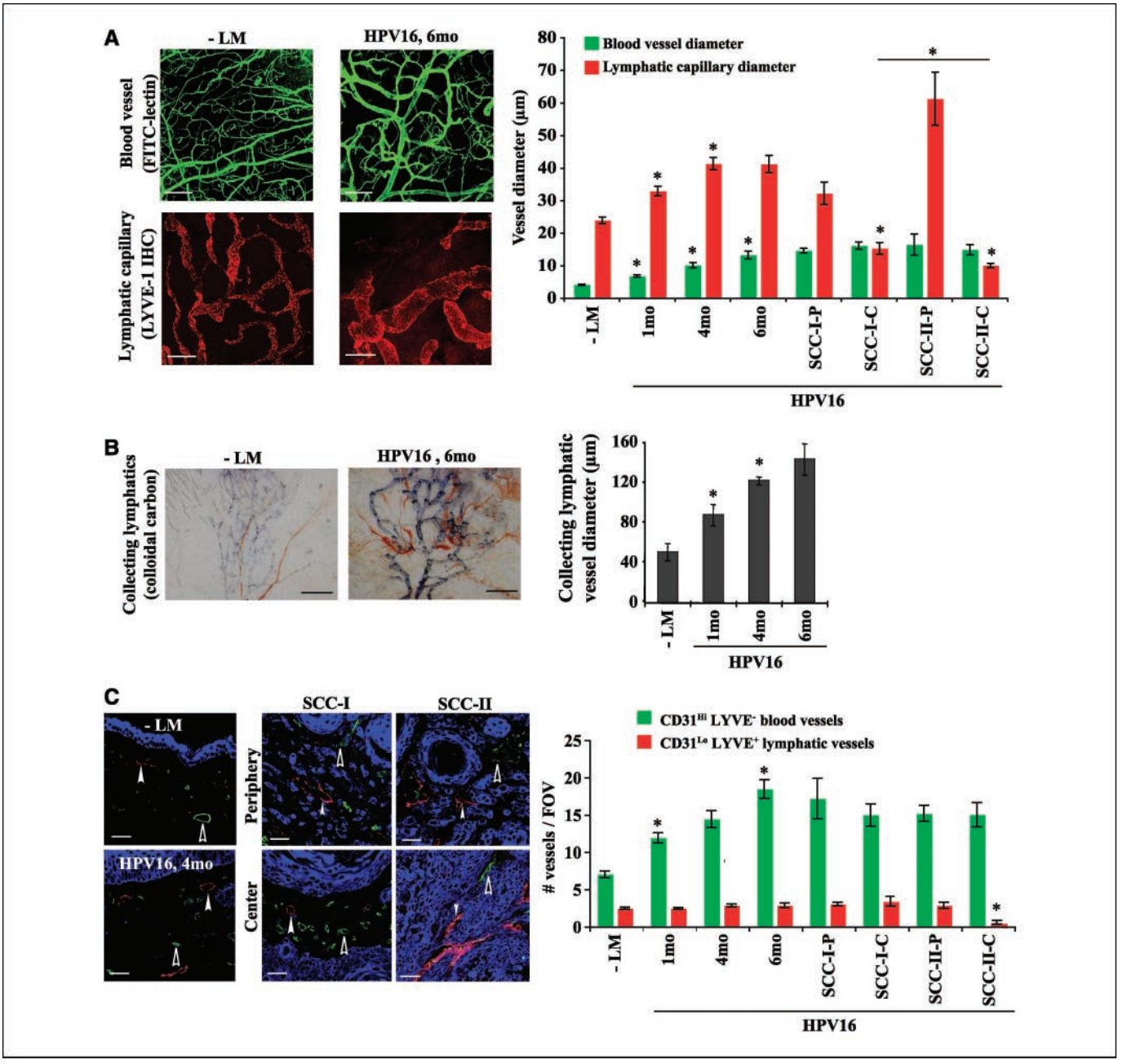

Figure 3. Vessel architecture and diameter during neoplastic progression. *A,* quantitative assessment of blood vessel diameter following fluorescent angiography (*green staining*) and lymphatic capillary diameter based on immunodetection of LYVE-1 (*red staining*) in whole-mount tissue sections of biopsies from −LM ear, premalignant ear, and carcinomas. *IHC,* immunohistochemistry. *Bar,* 50 μm. *B,* assessment of collecting lymphatic vessels following intralymphatic perfusion with colloidal carbon (*black staining*). *Red-colored vessels,* reveal visibility of blood vessels. *Bar,* 1.0 mm. *C,* quantitative analysis of vessel density of $CD31^{Hi}$/LYVE-1^{-} blood (*open arrows*) and $CD31^{Lo}$/LYVE-1^{+} lymphatic (*solid arrows*) vessel structures per random microscopic field of vision (*FOV*) in −LM, premalignant, and tumor tissue. *Bar,* 50 μm. *, $P \leq 0.05$, two-tailed unpaired nonparametric Mann-Whitney *U* test.

conjugated to the fluorophore tetramethylrhodamine isothiocyanate (TRITC; 10 mg/mL in PBS; Sigma) following i.d. injection. Drainage of TRITC-dextran was monitored in −LM ($n = 6$) and 4-month HPV16 ($n = 6$) ears over 20 min using a LUMAR microscope.

Results

Endothelial composition of blood and lymphatic vasculature during neoplastic progression. To evaluate the endothelial cell composition of premalignant versus malignant tissues (skin) during squamous carcinogenesis, we determined the relative percentage of endothelial cells at each stage of neoplastic progression in HPV16 mice, compared with wild-type negative littermate (−LM) controls, by assessing expression of the pan-endothelial cell adhesion molecule CD31 (25), present on both VECs and LECs, albeit at varying levels of expression (26), using flow cytometry of single cell suspensions (Fig. 1*A*). We found a progressive increase in the relative percentage of $CD31^{+}$ endothelial cells at each distinct premalignant stage (Fig. 1*A*). Carcinomas on the other hand contained a lower percentage of endothelial cells by comparison with premalignant tissues

(Fig. 1*A*), although tumor vasculature possessed angiogenic characteristics (16).

To determine the hematogenous versus lymphatic composition of these endothelial cell populations, we further used flow cytometry of single cell suspensions and quantitatively examined endothelial cells expressing CD31 and the mucin-type transmembrane glycoprotein podoplanin (Fig. 1*B*). Histochemically, podoplanin-positive endothelial cells are also LYVE-1 positive (Fig. 1*C*) and thus allow distinction between $CD31^{Hi}$/podoplanin^{-} VECs from $CD31^{Lo}$/podoplanin^{+} LECs. The average difference in CD31 signal intensity between $CD31^{Hi}$ VECs and $CD31^{Lo}$ LECs was 28%. A progressive increase in both VEC and LEC populations was found during premalignant progression; however, at the tumor stage, VECs represented a larger proportion of the total $CD31^{+}$ cells (Fig. 1*B*).

To determine the degree to which VECs and LECs exhibited proliferative behavior at each neoplastic stage, we evaluated endothelial cell proliferation in blood and lymphatic vessels as a function of BrdUrd incorporation. BrdUrd analysis of endothelial cells was limited to vessels with open lumina so as to distinguish BrdUrd-positive endothelial cells [whose nuclei protrude into vessel lumina (Fig. 2*A*)] from BrdUrd-positive mural cells (whose nuclei protrude toward interstitia). BrdUrd-positive $CD31^{Hi}$/LYVE-1^{-} VECs and $CD31^{Lo}$/LYVE-1^{+} LECs were not observed in skin sections of −LM mice, reflecting the quiescent nature of endothelial cells in homeostatic tissue (Fig. 2*B*). In contrast, BrdUrd-positive VECs and LECs were readily identified throughout premalignant progression albeit with distinct profiles (Fig. 2*A* and *B*). Whereas BrdUrd-positive VEC percentages steadily increased at each distinct premalignant stage (Fig. 2*B*), percentages of premalignant BrdUrd-positive LECs remained constant, albeit higher than in −LM skin (Fig. 2*B*). In carcinomas, open lumen blood and lymphatic vessels possessed distinct BrdUrd-positive endothelial cell distributions dependent on (*a*) SCC grade (lower-grade, well-differentiated SCC-I versus higher-grade, moderate-poorly differentiated SCC-II) and (*b*) SCC location (tumor center

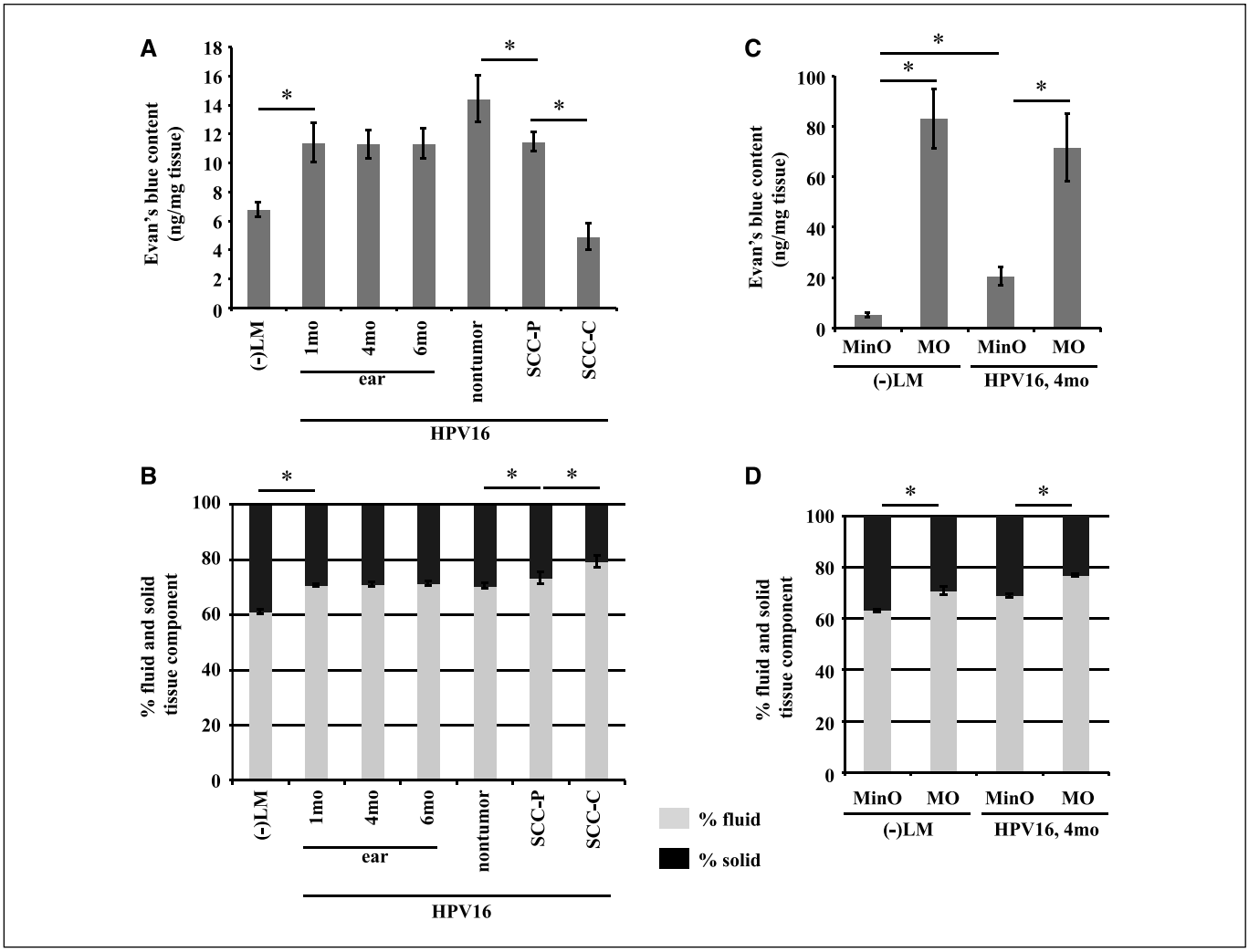

Figure 4. Functional interdependence of blood and lymphatic vessels during neoplastic progression. *A,* plasma protein extravasation and blood vessel leakiness in −LM, premalignant, and tumor tissue as assessed by Miles assay. *B,* quantification of tissue fluid in −LM, premalignant, and tumor tissue. *C,* induction of acute vascular leakage following mustard oil (*MO*) application in ear tissue of −LM and dysplastic HPV16 mice results in significantly increased vascular leakage, 16.5-fold ($P \leq 0.0001$, two-tailed unpaired nonparametric Mann-Whitney *U* test) and 3.5-fold ($P = 0.0043$, two-tailed unpaired nonparametric Mann-Whitney *U* test), respectively. *MinO,* mineral oil. *D,* mustard oil–induced leakage results in significantly increased tissue fluid in −LM controls (8.0%) and premalignant tissue (8.0%).
*, $P \leq 0.05$, two-tailed unpaired nonparametric Mann-Whitney *U* test.

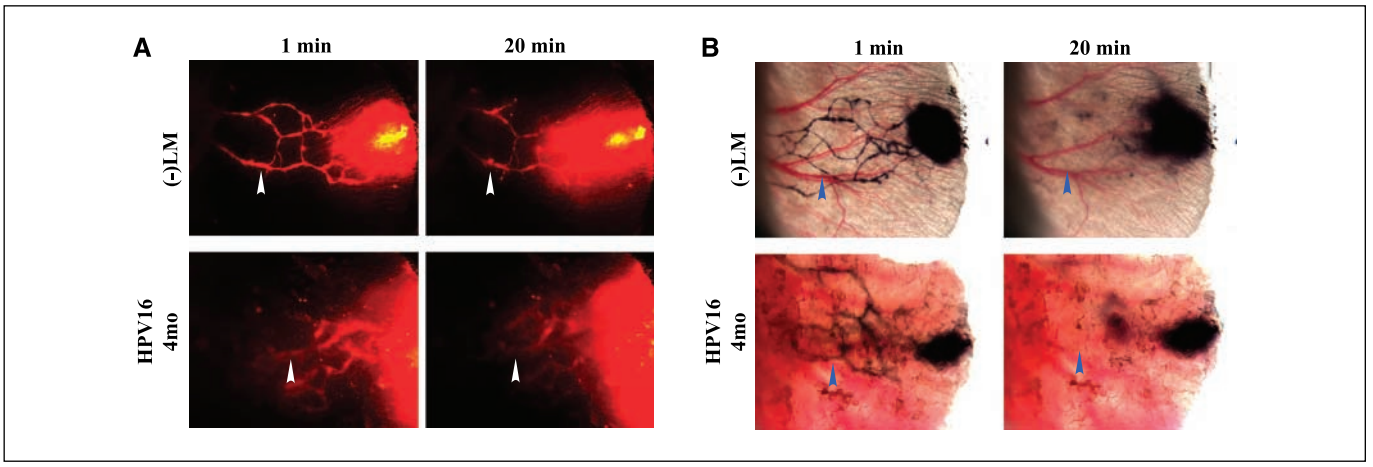

Figure 5. Functionality of lymphatic vasculature during premalignant progression. *A*, functionality of initial lymphatics (*arrows*) was assessed following i.d. injection of high molecular weight (2 MDa) TRITC-conjugated (*red staining*) dextran in −LM and dysplastic HPV16 ears and clearance monitoring over a 20-min time period. *B*, draining capability of collecting lymphatic vessels in −LM and dysplastic HPV16 ear tissue was assessed by intralymphatic injection of colloidal carbon (*black staining*) and clearance monitoring over 20 min. Colloidal carbon in the lymphatic vasculature (*arrow*) was completely drained after 20 min.

versus tumor periphery). Open lumen blood and lymphatic vessels were identified at peripheries of both SCC-I and SCC-II carcinomas and in centers of SCC-I (Fig. 2*A* and *B*). In the center of SCC-IIs, open lumens were only found in blood vessels and not in lymphatic vessels (Fig. 2*B*). Analysis of proliferating endothelial cells in vessels with open lumens in carcinomas revealed an increase in both BrdUrd-positive VECs and LECs (Fig. 2*B*) compared with precursor premalignant lesions. VECs and LECs both evidenced increased presence of BrdUrd-positive endothelial cells in tumor centers and peripheries of SCC-I (Fig. 2*A* and *B*); however, in SCC-II, proliferating VECs were found in tumor centers and in the periphery, whereas proliferating LECs were limited to tumor periphery (Fig. 2*A* and *B*).

Blood and lymphatic vessel density and diameter during neoplastic progression. To determine whether increased proliferation of endothelial cells in blood or lymphatic vessels yielded increased vessel densities and sprouting of new vessels or alternatively only resulted in increased girth (diameter) of preexisting vessels, we assessed blood and lymphatic vessel diameters and density (number of vessels per random high-power field of vision) at each neoplastic stage (Fig. 3). Using fluorescent angiography to assess blood vessel diameter in −LM and histopathologically staged neoplastic lesions, we found that blood vessel diameters significantly increased at each premalignant stage (Fig. 3*A*). In SCC-I and SCC-IIs, blood vessel diameter was similar in both tumor centers and periphery (Fig. 3*A*). Assessment of LYVE-1$^+$ lymphatic capillary diameter similarly revealed a progressive increase during premalignant progression, albeit less dramatic compared with blood vessels (Fig. 3*A*). Because collecting lymphatic vessels express LYVE-1 at low levels, we used intralymphatic perfusion of colloidal carbon to assess their diameter in −LM and premalignant tissue and observed a progressive increase in diameter in premalignant tissue (Fig. 3*B*). Lymphatic vessel diameters in carcinomas at SCC-I peripheries were similar to late dysplastic tissues, whereas lymphatic vessels at SCC-II peripheries were further dilated (Fig. 3*A*). LYVE-1$^+$ lymphatic vessel diameters in SCC-I centers were decreased by comparison with peripheries but were also significantly greater than those found in the center of SCC-IIs (Fig. 3*A*), thus indicating that proliferation of endothelial cells results in vessels with enhanced diameters in both vessel systems in premalignant and SCC tissues.

To evaluate the sprouting activity of new vessels as a result of increased VEC and/or LEC proliferation, we assessed vessel densities by quantitatively examining CD31^{Hi}/LYVE-1$^-$ (blood) and CD31^{Lo}/LYVE-1$^+$ (lymphatic) vessels in tissue sections (Fig. 3*C*). We found significant increases in the number of CD31^{Hi}/LYVE-1$^-$ blood vessels per field of vision at each premalignant stage (Fig. 3*C*). In carcinomas, the number of CD31^{Hi}/LYVE-1$^-$ blood vessels remained comparable with late dysplastic tissue independent of localization or SCC grade (Fig. 3*C*). In contrast, the progressive increase in lymphatic vessel diameter was not accompanied by increased vessel density as shown by constant numbers of CD31^{Lo}/LYVE-1$^+$ lymphatic vessels in premalignant tissues and in periphery and centers of SCC-I and periphery of SCC-IIs (Fig. 3*C*). CD31^{Lo}/LYVE-1$^+$ lymphatic vessels were decreased in SCC-II centers (Fig. 3*C*). Together, these data indicate that proliferation of endothelial cells in the two vessel systems results in distinct phenotypic responses. Whereas blood vessels increase in diameter and density, lymphatic vessels increase only in diameter without an increase in density, thus reflecting increased sprouting of new blood vessels (i.e., classic angiogenesis) as opposed to increased girth without sprouting of lymphatic vessels.

Altered tissue fluid homeostasis and lymphatic functionality in neoplastic tissue. Altered proliferation and/or diameter of either vessel system could variably alter their physiologic functions in tissue and thereby alter tissue hemodynamics and/or fluid content. To assess functionality of blood vessels, we evaluated their perfusability on fluorescein-conjugated *L. esculentum* injection (Fig. 3*A*) and their leakage characteristics using a modified Miles assay (Fig. 4*A*; ref. 27). These studies revealed increased extravasation of Evans blue tracer in hyperplastic and dysplastic HPV16 tissue compared with −LM skin (Fig. 4*A*). In spite of the increased density and diameter of perfused, thus functional blood vessels in tumor tissue (Fig. 3*A* and *C*), we found decreased vascular leakage in carcinomas where distinct extravasation characteristics were found in tumor centers versus periphery (Fig. 4*A*).

To examine whether increased extravasation of plasma proteins resulted in increased tissue fluid content, we evaluated the

relationship between blood vessel leakage (Fig. 4*A*) and lymphatic drainage by determining tissue fluid amounts at each neoplastic stage (Fig. 4*B*) and found that tissue fluid in premalignant HPV16 ear tissue was significantly higher than −LM tissue and further increased in tumor peripheries, with the central regions of SCCs having the highest fluid content (Fig. 4*B*). Because a major function of lymphatics is to drain excess tissue fluid, one interpretation of these data would be that the drainage capacity of premalignant lymphatics had been exceeded due to lymphatic dysfunction perhaps reflected by tissue fluid accumulation. To access this hypothesis, we further induced vascular leakage acutely by topical application of mustard oil (compared with mineral oil) and assessed Evans blue dye leakage into dysplastic HPV16 ear skin versus −LM. As expected, mustard oil application further increased leakage of plasma proteins out of blood vessels in both −LM and HPV16 skin, 16.5- and 3.5-fold, respectively (Fig. 4*C*); however, these increases resulted in similar accumulations of tissue fluid content in −LM (8%) and dysplastic HPV16 (8%) tissue (Fig. 4*D*), indicating that clearance of tissue fluid by premalignant lymphatics is not at maximum capacity. Thus, whereas lymphatics in acutely activated homeostatic tissue are more efficient at clearing excess tissue fluid, functional lymphatics in premalignant tissue maintain tissue fluid dynamics to a similar degree.

To directly assess lymphatic functionality in premalignant tissue, we i.d. injected high molecular weight, fluorescently labeled dextran (TRITC-dextran) tracer and monitored uptake and drainage kinetics by lymphatic capillaries over time in −LM and dysplastic ear skin (Fig. 5*A*). Lymphatic capillaries were rapidly labeled by tracer in both control and HPV16 ears, indicating rapid uptake. Twenty minutes following injection, tracer-containing capillaries were detectable, indicating continuous lymph formation, thus showing their functional status.

Functionality of collecting lymphatic vessels in premalignant tissue was also evaluated following intralymphatic injection of colloidal carbon (Fig. 5*B*). Colloidal carbon is excluded by lymphatic capillaries during lymph formation due to its large molecular weight.[4] Direct injection of colloidal carbon into dermal lymphatics of −LM and dysplastic ear skin revealed efficient drainage of tracer from collecting lymphatic vessels in both tissues within 20 min (Fig. 5*B*), thus showing their similar functionality. Together, these analyses reveal functionality of lymphatic capillaries and collecting vessels in premalignant tissues based on their ability to efficiently clear tracer and fluid (Fig. 5*A* and *B*) through their open lumina (Fig. 3*A* and *B*). Direct analysis of lymphatic functionality in tumor tissue was hampered by technical difficulties doing direct intralymphatic colloidal carbon injection as well as analysis of interstitial drainage using smaller molecular weight dyes (colorimetric or fluorescent) readily taken up by functional lymphatics. Despite these failed attempts to analyze lymphatic functionality in tumor tissue, the implication that open luminal status is associated with vessel functionality in premalignant tissue, in combination with our assessment of tissue fluid content in tumor centers versus tumor periphery (Fig. 4*B*), leads us to conclude that lymphatics with open lumina in premalignant tissue, at peripheries of SCCs and centers of grade 1 SCCs (Fig. 3*C*), retain functionality where they efficiently clear interstitial fluid, as opposed to tumor centers of less-differentiated SCCs where lymphatic lumens are predominantly compressed and exhibit diminished fluid drainage capacity (Fig. 3*C, bottom right*), a characteristic feature of poorly differentiated carcinomas (28–30).

[4] J. Nagy, personal communication.

Discussion

Data presented herein indicate that blood and lymphatic vasculature undergo distinct physiologic and architectural alterations during *de novo* neoplastic progression accompanying carcinoma development. Whereas endothelial cells of each network acquire proliferative capabilities as neoplasia ensues, resultant phenotypes of blood versus lymphatic vessels are distinct. Whereas blood vessels undergo classic angiogenic changes (e.g., increased endothelial cell proliferation, vessel diameter, density, and leakage), lymphatic vessels evidence no alterations to support the notion that sprouting of new vessels occurs. Instead, the increased level of proliferating LECs present in lymphatic vessels enhances diameter of vessels that efficiently clear interstitial fluid in premalignant tissues and peripheries of carcinomas, but not in their centers, areas where interstitial fluid content remains high. Together, these studies show that neoplasia-associated angiogenesis, as opposed to lymphangiogenesis, is a distinct vascular process even when initiated by similar physiologic stimuli.

Endothelial cell proliferation. Endothelial cells in quiescent tissues divide approximately once every 2 to 3 years if unstimulated (31). During cancer development, however, it is well established that local tissue levels of the proangiogenic factor vascular endothelial growth factor (VEGF)-A increase and, as a result, enhance proliferation of VECs and sprouting of new blood vessels from preexisting vascular beds (5). In contrast, lymphangiogenesis (e.g., induction of LEC proliferation) in human cancers (32, 33) correlates instead with increased tissue levels of VEGF-C and VEGF-D (34–36). Accordingly, exogenous expression of VEGF-C or VEGF-D in experimental murine tumor xenograft models induces LEC proliferation and subsequent lymphangiogenesis (37, 38), whereas exogenous VEGF-A expression induces not only an angiogenic response but also lymphangiogenesis in nontumor (24) and tumor tissue (39). It is not clear, however, if LEC responses to increased VEGF-A levels represent a direct intrinsic response of LECs or, instead, an indirect physiologic response resulting from increased tissue fluid due to increased leakage of plasma proteins from newly formed immature angiogenic blood vessels.

In this study, we found evidence of angiogenesis as well as lymphangiogenesis in premalignant tissues of HPV16 mice and in emergent malignant carcinomas. Patterns of LEC proliferation varied from that observed with VECs, thus indicating distinct responses between the two endothelial cell subpopulations to proangiogenic and/or prolymphangiogenic growth factors present in their microenvironment. Whereas VEGF-A mRNA levels have been reported to remain constant during premalignant angiogenesis in a UV-induced murine skin carcinoma model (40), enhanced VEC proliferation in HPV16 mice is paralleled by progressive increases in VEGF-A mRNA expression (41) and protein levels (42). About LEC proliferation, our analyses indicate that, although LECs in premalignant tissue evidence enhanced proliferative status compared with homeostatic tissue, their proliferation does not significantly increase until malignant conversion occurs. Because we have observed increased mRNA levels of VEGF-C in fully malignant carcinomas (data not shown), our interpretation of these data is that LECs respond modestly to increased VEGF-A levels in premalignant tissue but more are responding to increased

microenvironmental stress and need to clear fluid from premalignant tissue resultant from leaky immature angiogenic blood vessels.

Vascular sprouting. VEGF-A is a major proangiogenic factor well documented to induce VEC proliferation and vascular sprouting (43). Sprouting of the lymphatic vasculature, however, depends on VEGF-C in experimental chick chorioallantoic membrane assays, wound healing, and embryonic development (44–46). Recent studies in murine tumor models (transgenic and xenograft models) provide evidence that increased levels of VEGF-C and/or VEGF-D promote tumor lymphangiogenesis, including lymphatic vessel sprouting (47–50). In contrast to these experimental tumor models, no increase in lymphatic vessel density was observed in HPV16 tissues, indicating that LEC proliferation in a *de novo* tumor model lacking exogenous expression of VEGF-C and/or VEGF-D does not result in lymphatic vessel sprouting but instead contributed to increased diameter (girth) of existing lymphatics to enable rapid clearance of tissue fluid resulting from increased leakage of plasma proteins from angiogenic blood vessels.

Tissue fluid dynamics in premalignant and SCC tissue. Blood and lymphatic vessels are functionally interdependent vascular systems (6) that together regulate tissue fluid dynamics and influence tissue homeostasis. In a UV-induced skin carcinogenesis model, Hagendoorn et al. (40) revealed alterations in blood vasculature, architectural changes, and impairment of lymphatic vessels along with increased IFP in premalignant tissues and thus hypothesized that blood and lymphatic vasculature loose their functional interdependence during premalignant progression. Our data, however, indicate that, during squamous carcinogenesis in HPV16 mice, the two vessel systems retain a functional interrelationship in premalignant tissue and in well-differentiated carcinomas as evidenced by increased blood vessel leakage accompanied by retained ability to clear fluid, albeit in tissue with higher tissue fluid content than normal.

Lymphatic vessel functionality. We observed that intratumoral lymphatic vessel functionality inversely correlates with SCC grade. In the published literature, there are contradicting reports on the existence of intratumoral lymphatics in human (9, 10, 32, 33, 51) and experimental rodent (37, 38, 40, 49, 52) tumors, where only peripheral lymphatic structures are hypothesized to retain functionality, whereas intratumoral lymphatic structures were rendered nonfunctional due to compression by rapidly proliferating tumor cells (52). Similarly, we found functional lymphatic vessels at tumor peripheries but also observed open lumen/functional lymphatic vessels in centers of low-grade SCCs. Our interpretation of these observations is that functional properties of higher-grade, less-differentiated carcinomas correlate with, and is effected by, limited lymphatic vessel functionality that, in turn, likely regulates tumor cell physiology and/or behavior.

In summary, by examining proliferation, diameter, density, and functionality of blood versus lymphatic vasculature during *de novo* cancer development, we have revealed unique features of each vasculature that illuminates their interdependency during premalignant progression as opposed to their independence in higher-grade carcinomas. Maintenance of the interdependence between blood and lymphatic systems during premalignancy and in low-grade carcinomas enables efficient fluid transport from leaky blood vasculature via interstitia into draining, open lumen lymphatic vessels, which would result in minimizing IFP. The closed nature of lymphatic vessels in higher-grade carcinomas would exclude this and support elevated IFPs common to less-differentiated carcinomas. The implication of these findings supports the notion that delivery of anticancer therapeutics would best be realized in premalignant tissue, *in situ* carcinomas, or carcinomas displaying well-differentiated characteristics, where tissue hemodynamics better support drug delivery.

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Research article

The initial steps of ovarian cancer cell metastasis are mediated by MMP-2 cleavage of vitronectin and fibronectin

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Most patients (80%) with ovarian cancer (OvCa) present with metastatic disease. Attachment of OvCa cells to peritoneum and omentum represents the first rate-limiting step for metastatic spread. Therefore, identifying factors regulating cell attachment in the abdominal cavity is critical to the development of therapeutic agents. We show here that MMP-2 expression was upregulated in OvCa cells upon attachment to their microenvironment. Downregulation of MMP-2 mRNA or pharmacological inhibition of MMP-2 proteolytic function, in both human OvCa primary cells and cell lines, reduced attachment of OvCa cells to a 3D organotypic model of metastatic OvCa, full human omentum or peritoneum, and in vivo to mouse peritoneum and omentum. Absence of MMP-2 in the host did not alter OvCa adhesion, as determined utilizing mice harboring homozygous null mutations in either the *Mmp2* or *Mmp9* genes. Conversely, adhesion induced upregulation of MMP-2 mRNA in OvCa cells. MMP-2 inhibition in OvCa cells through pharmacological or antibody treatment prior to i.p. dissemination in nude mice significantly decreased tumor growth and metastasis and extended survival. MMP-2 enhanced peritoneal adhesion of OvCa cells through cleavage of ECM proteins fibronectin (FN) and vitronectin (Vn) into small fragments and increased binding of OvCa cells to these FN and Vn fragments and their receptors, $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrin. These findings indicate that MMP-2 expressed by metastatic OvCa cells functionally regulates their attachment to peritoneal surfaces.

Introduction

Ovarian cancer (OvCa) has the highest mortality rate of all gynecologic tumors and is the fifth leading cause of cancer death among US women (1). It is predominantly confined within the abdominal cavity and, unlike breast, colon, or lung cancer, rarely metastasizes hematogenously. Once an ovarian epithelial cell undergoes neoplastic transformation, it freely disseminates throughout the peritoneal cavity, carried by peritoneal fluid that facilitates attachment to peritoneum and omentum. The omentum is a large fat pad (approximately 12 × 12 cm) located inferior to the stomach and draped over the small bowel. It is the most common metastatic site (80%) for OvCa cells (2) followed by implants on the abdominal peritoneum. Identification of cofactors regulating OvCa cell attachment to omentum and/or peritoneum would have tremendous clinical utility, by enabling identification of cellular or molecular targets that could be pursued therapeutically and thus, enabling blockade of a critical step necessary for OvCa metastasis within the peritoneal cavity.

A role for MMPs in OvCa development has been postulated based upon the observation that several members of the MMP family are upregulated during OvCa neoplastic progression (3). When MMPs were first characterized (4), it was hypothesized that their major contribution to cancer development was merely to degrade ECM molecules, thereby facilitating cancer cell migration/invasion across tissue boundaries. More recent insights have, however, defined a more complex role for MMPs in cancer. They are now recognized as key regulators of various neoplastic processes by virtue of their ability to mediate differentiation, proliferation, and survival of neoplastic cells (5), release mitogenic growth factors from cell surfaces and from ECM reservoirs, and regulate tumor-associated angiogenesis (6, 7). In spite of these revelations, no MMPs have been identified as being absolutely required for neoplastic cell migration/invasion into ectopic tissue compartments in vivo. Based upon their perceived importance as mediators of ECM remodeling, clinical trials assessing efficacy of broad spectrum MMP inhibitors (MMPI) in patients with solid tumors, including non–small cell lung (8), pancreatic (9), gastric (10), and OvCas (11), were undertaken in patients with recurrent, metastatic, chemotherapy-resistant tumors. Unfortunately, none of the MMPI evaluated improved patient survival (12).

Interestingly, several groups using preclinical mouse models of de novo cancer development (7, 13) revealed that MMPI efficacy may be best achieved during earlier stages of tumor development, prior to appearance of bulky and/or metastatic disease. In the RIP1-Tag2 model of pancreatic islet carcinogenesis (7), tumor burden was significantly reduced in tumor-prone mice when mice were treated with the MMPI batimastat during early neoplasia, prior to malignant conversion and development of islet adenocarcinomas. Moreover, if tumor-prone mice were treated later in their disease progression, when tumors were already present, there was no significant effect (14). Similarly, growth of OvCa xenografts is significantly diminished if mice are treated with batimastat immediately following tumor cell injection; whereas, if batima-

Nonstandard abbreviations used: APMA, aminophenylmercuric acetate; FACS, fluorescent-activated cell sorting; FN, fibronectin; HPF, human primary fibroblast; HPMC, human primary mesothelial cell; MMPI, MMP inhibitor; MT1, membrane-type 1; OvCa, ovarian cancer; Vn, vitronectin.

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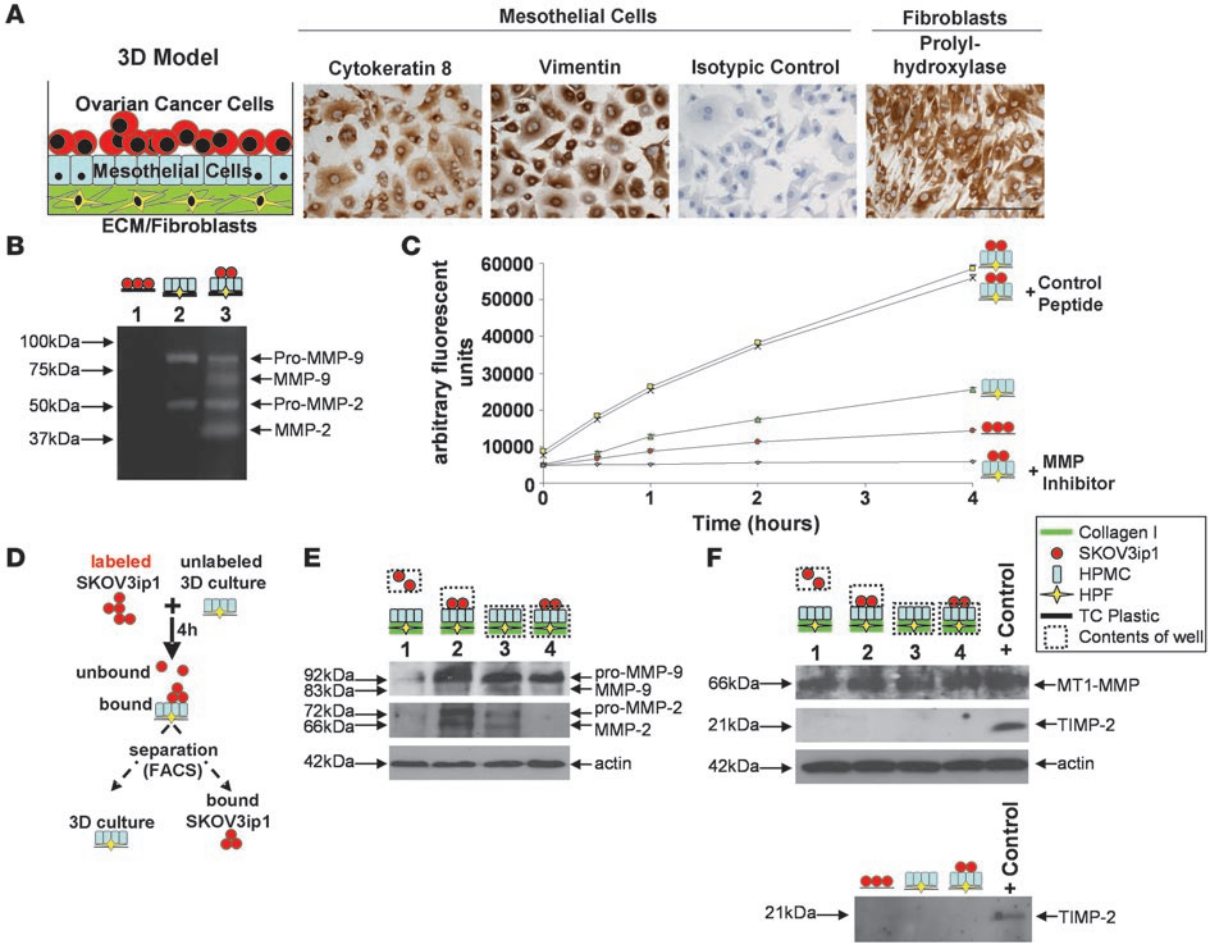

Figure 1

Binding of OvCa cells to an omental 3D culture increases MMP-2/-9 expression and secretion in cancer cells. (**A**) Concept for a 3D culture to imitate abdominal mesothelium. HPMCs stained with an antibody against cytokeratin 8, vimentin, or an isotypic specific control antibody. HPFs stained with a prolyl-hydroxylase antibody recognizing human fibroblasts. Scale bar: 50 μM. (**B**) Zymogram. Conditioned media from SKOV3ip1 cells (red circles), the 3D culture (composed of HPMCs [blue rectangles], HPFs [yellow diamonds], and collagen I [green rectangles]), or coculture was subjected to gelatin zymography. (**C**) Gelatinase assay. Cell-associated gelatinolytic activity in the indicated cell populations, with or without an MMP-2/-9 inhibitor, was determined using a quenched fluorogenic peptide. Fluorescence was measured with a fluorescence spectrophotometer. (**D**) Schematic of adhesion assay and subsequent cell sorting. Fluorescently labeled SKOV3ip1 cells were mixed with 3D culture for 4 h. The cells were mechanically removed from the plate and sorted by FACS. (**E**) Cell extracts were subjected to immunoblotting using an MMP-2 and MMP-9 specific antibody. The membrane was reprobed with an antibody against actin. Lane 1, unbound, floating SKOV3ip1 cells; lane 2, SKOV3ip1 cells that had attached to the 3D culture after FACS; Lane 3, 3D culture alone; Lane 4, 3D culture that had bound SKOV3ip1 cells after FACS. (**F**) Western blot for MT1-MMP and TIMP-2. SKOV3ip1 were plated on the 3D culture and then sorted as indicated in **D**. Cell extracts (top panel) or conditioned media (bottom panel) were subjected to immunoblotting with MT1-MMP or TIMP-2 antibody. HT-1080 CM was used as positive control.

stat is administered after solid tumors are established, minimal efficacy is achieved (13). Similar results have also been reported with gastric cancer and platinum-resistant OvCa xenografts (15, 16), indicating that efficacy of MMPI therapy is most significant when administered early in disease progression. Furthermore, in the clinical arena, it has now been reported that treatment of early-stage cancer with an MMP-2/-9 inhibitor (marimastat) might increase survival (9). We previously examined expression of MMP-2 and MMP-9 in human OvCa tissue and found that essentially all invasive OvCas, including early stage I cancers as well as metastatic implants, overexpress both MMP-2 and MMP-9, while normal ovarian tissue exhibits significantly lower levels of expression (17, 18), indicating that MMP-2 and MMP-9 are upregulated early in OvCa progression.

Given these findings, we hypothesized that type IV collagenases, e.g., MMP-2/-9, might be important regulators for early steps of OvCa metastasis. We report here that upregulation of MMP-2 in OvCa cells is critical for their adhesion to the mesothelial lining of the peritoneum and omentum. Using an orthotopic model of OvCa cell metastasis, we found that early inhibition of MMP-2 reduced ovarian tumor cell adhesion and metastasis and thus, significantly prolonged survival of experimental mice. Subsequent or repeated treatment of solid tumors with an MMP-2 inhibitor minimally reduced ovarian tumor metastasis and had no effect on

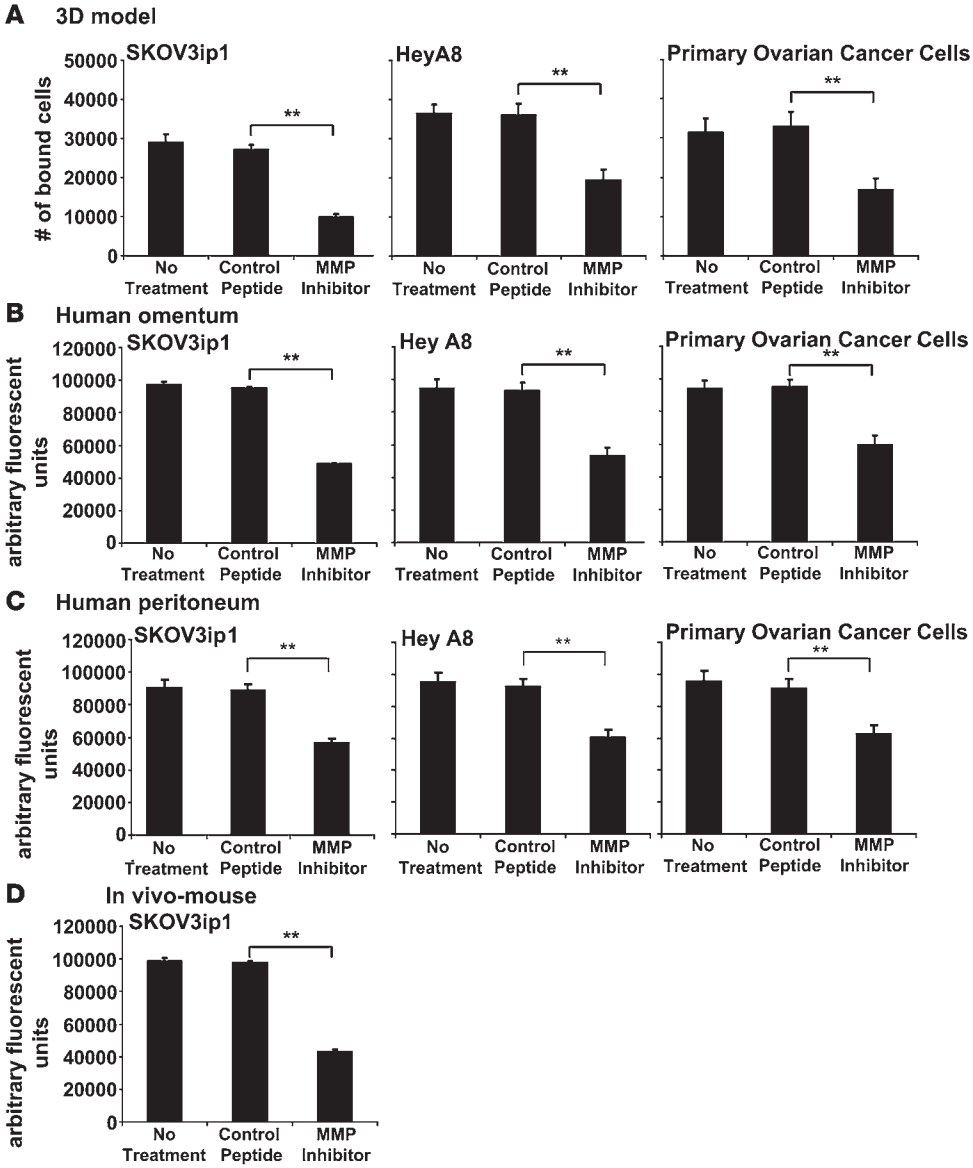

Figure 2
An MMPI inhibits adhesion of OvCa cells to a 3D culture of human omentum, full human omentum and peritoneum, and mouse omentum. SKOV3ip1, HeyA8, or primary human OvCa cells (50,000) were fluorescently labeled and added to the 3D culture (**A**), a piece of full human omentum (**B**), or full human peritoneum (**C**). The 3D culture, the human omentum, and the human peritoneum were washed with PBS, adherent cells on the omentum were lysed with NP-40, and fluorescence intensity was measured with a fluorescence spectrophotometer. (**D**) SKOV3ip1 cells were fluorescently labeled and 4×10^6 were cells injected i.p. into nude mice ($n = 3$). The mice were sacrificed after 4 hours, omentum and peritoneum were lysed with NP-40, and fluorescence was measured. $^{**}P < 0.001$. Each bar represents the mean of 3 wells and SD. Each graph is representative of at least 3 independent experiments.

survival. MMP-2 increased the adhesive capability of OvCa cells by specific cleavage of fibronectin (FN) and vitronectin (Vn), allowing for enhanced attachment of OvCa cells to FN and Vn fragments through $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrin. Therefore, our findings implicate MMP-2 in OvCa adhesion and indicate that therapeutic efficacy of MMP-2–selective inhibitors will be best achieved clinically if applied prior to peritoneal dissemination.

Results

Coculture of OvCa cells with mesothelial and stromal cells induces MMP-2 and MMP-9. The peritoneal cavity, including the omentum, is covered by mesothelium consisting of a confluent layer of epithelial mesothelial cells, lying juxtaposed to a layer of ECM where fibroblasts are embedded. Because peritoneal surfaces are the most common site of OvCa metastases (2), we established what we believe to be a novel organotypic 3D coculture model mimicking human omentum, in order to examine the role of MMP-2 and MMP-9 in adhesion (Figure 1A). Collagen I was used as the principal ECM molecule, because a dense type I collagen matrix is present in the omental and peritoneal basement membrane and because OvCa cells preferentially adhere to collagen I (19). Human primary mesothelial cells (HPMCs) and human primary fibroblasts (HPFs) were extracted from human omentum and maintained in culture for 2 passages and then evaluated for appropriate expression of cell type–specific markers in HPMCs (e.g., cytokeratin 8) and in HPFs (e.g., vimentin and proline-4-hydroxylase) (20) (Figure 1A). To analyze the role of MMP-2 and MMP-9 in adhesion, the OvCa cell line, SKOV3ip1 that neither expresses nor secretes MMP-2 or MMP-9, was cocultured with fibroblasts and HPMCs, both of which secrete pro–MMP-2 and pro–MMP-9 (21). Coculture led to a significant conversion of the pro form of MMP-2 and MMP-9 to their enzymatically active forms (Figure 1B). This was paralleled by an increase in cell surface gelatinolytic activity once SKOV3ip1 cells were added to 3D cocultures (SKOV3ip1 alone, V_{max} 2,374 U/h; SKOV3ip1 cocultured, V_{max} 12,436 U/h; $P < 0.0005$). Preincubation with an MMP

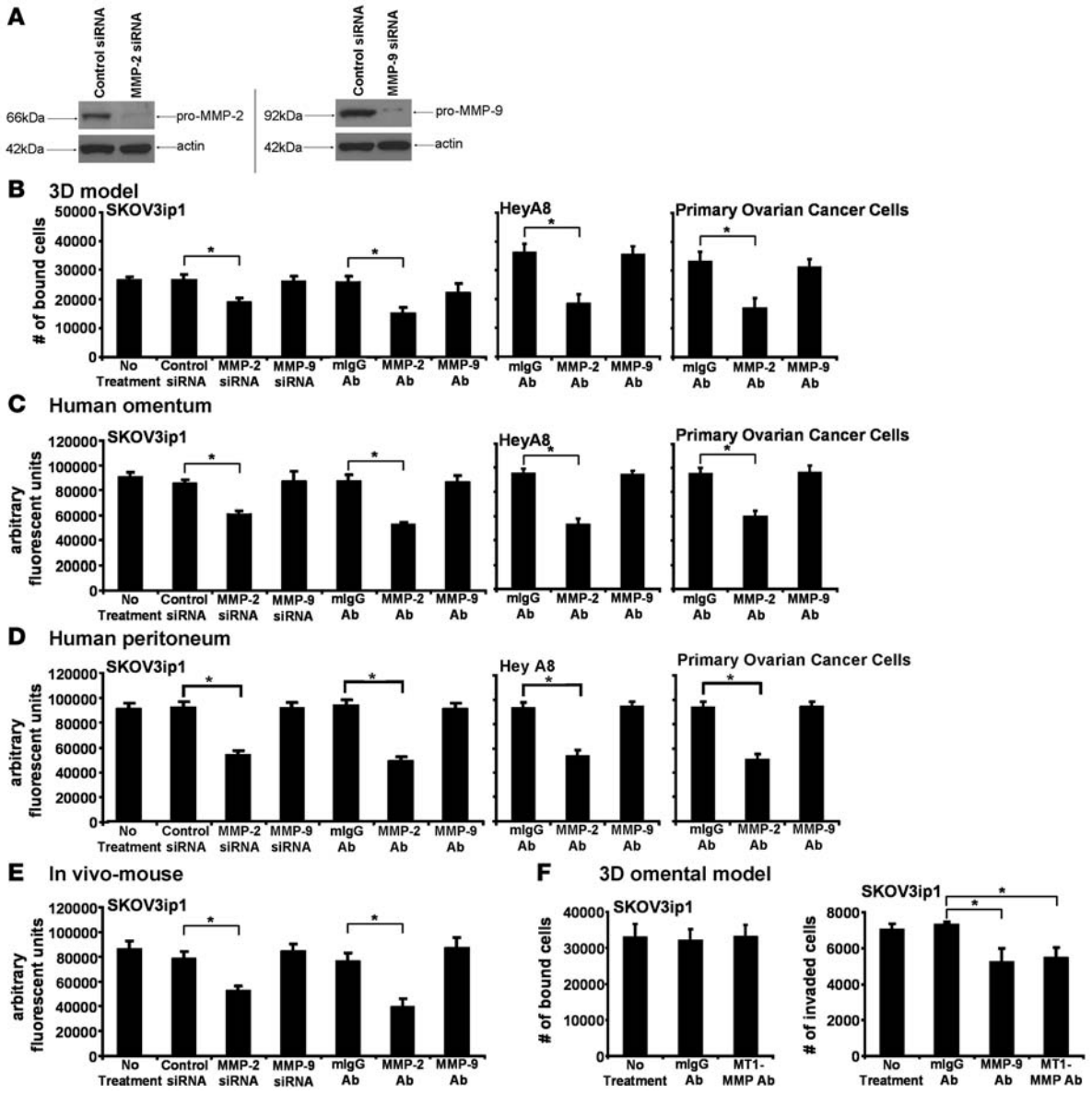

Figure 3

Attachment of OvCa cells depends on MMP-2. (**A**) SKOV3ip1 cells were transfected with a siRNA specific for MMP-2, MMP-9, or a control siRNA and plated on collagen type I, and MMP-2 and MMP-9 were detected by immunoblotting with specific antibodies. The membrane was reprobed with actin. (**B**–**D**) Adhesion assays. SKOV3ip1 cells were transfected with a siRNA specific for MMP-2 and MMP-9. SKOV3ip1, HeyA8, and primary OvCa cells were pretreated with an MMP-2– or MMP-9–blocking antibody and then fluorescently labeled. OvCa cells were added to the 3D culture (**B**), a piece of full human omentum (**C**), or full human peritoneum (**D**), and the adherent cells quantified with a fluorescence reader as described in Figure 2. (**E**) SKOV3ip1 cells (1×10^6) were labeled fluorescently and injected i.p. into nude mice for 4 hours ($n = 3$). Mice were sacrificed after 4 hours, omentum and peritoneum was lysed with NP-40, and fluorescence was measured. (**F**) Adhesion assay. SKOV3ip1 cells (5×10^4) pretreated with the MT1-MMP antibody were added to the 3D culture and an adhesion assay performed as described in Figure 2 (left panel). Invasion assay (right panel). SKOV3ip1 cells (3×10^4) were treated with a MT1-MMP– or MMP-9–blocking antibody, and invasion was analyzed after 24 hours. *$P < 0.01$.

inhibitory peptide that binds active sites of both MMP-2 and MMP-9 with high specificity and blocks catalytic activity (22, 23) inhibited cell surface gelatinolytic activity, while the scrambled control peptide had no effect (Figure 1C), thus, implicating gelatinolytic activity during OvCa cell adhesion.

To determine which cell population was necessary for the increased presence of MMP-2/-9, SKOV3ip1 cells were fluorescently labeled and plated on the 3D culture. Four hours later, cells were isolated by flow cytometry and immunoblotted to assess MMP-2 and MMP-9 levels (Figure 1D). Unbound SKOV3ip1 cells expressed minimal pro–MMP-2 and pro–MMP-9 protein; however, upon attachment to the 3D culture, both the pro and active form of MMP-2 and the pro form of MMP-9 were induced. The 3D culture, consisting of HPMCs and HPFs, expressed minimal

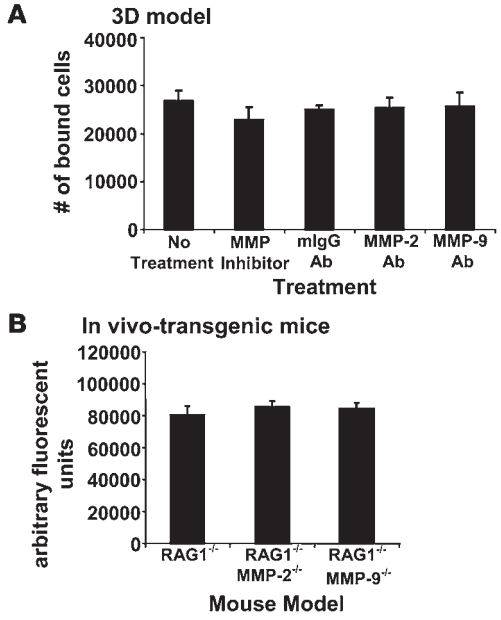

Figure 4

Host-derived MMP-2 and MMP-9 does not affect adhesion of OvCa cells. (**A**) The 3D culture was pretreated with either the cyclic peptide inhibiting preferentially MMP-2/-9 or a monoclonal antibody against MMP-9 or MMP-2, respectively. Subsequently, adhesion assay was performed as described in Figure 2. (**B**) Six-week-old *RAG-1*$^{-/-}$ mice, WT for *MMP2* and *MMP9* or *RAG-1*$^{-/-}$ either deficient in *MMP2* (*MMP2*$^{-/-}$) or *MMP9* (*MMP9*$^{-/-}$), were injected with fluorescently labeled SKOV3ip1. After 4 h, the mice were sacrificed, equal parts of omentum and peritoneum were lysed in NP-40, and fluorescence was measured.

MMP-2, while a constitutively high level of MMP-9, independent of cancer cell attachment, was prominent (Figure 1E).

Matrix remodeling activity of MMP-2 is the result of a stoichiometric interaction between membrane-type 1–MMP/MMP-14 (MT1-MMP/MMP-14), a transmembrane receptor and proteolytic activator of pro–MMP-2 (3), and TIMP-2, a tissue inhibitor of metalloproteinase. Binding of SKOV3ip1 cells to the 3D culture did not alter MT1-MMP or TIMP-2 protein expression (Figure 1F). MT1-MMP was constitutively high in both the 3D culture and OvCa cells, while TIMP-2 was not secreted (Figure 1F, lower panel) or detected on the cell surface (Figure 1F, upper panel).

In view of these findings, we hypothesized that MMP-2 or MMP-9 mediated OvCa cell adhesion to mesothelium. To test this, we took a 3-pronged approach and evaluated attachment of 2 established OvCa cell lines (SKOV3ip1, HeyA8) and primary OvCa cells to the 3D coculture (Figure 2A) to full-thickness human omentum (Figure 2B) and peritoneum (Figure 2C) removed at surgery and to omentum and peritoneum within the abdominal cavity of immune-deficient mice. Incubation of SKOV3ip1, HeyA8, or primary OvCa cells with the MMP-2/-9–blocking peptide reduced cancer cell adhesion to the 3D coculture by 63%, 46%, and 43% ($P < 0.005$), respectively (Figure 2A). Confirming and extending these results, the MMP-2/-9 inhibiting peptide also reduced adhesion to the short-term coculture with human omentum (Figure 2B) and human peritoneum (Figure 2C). We then determined whether the peptide also inhibited in vivo adhesion of SKOV3ip1 cells to the peritoneal cavity of nude mice (Figure 2D). Adhesion was evaluated 4 hours following i.p. injection of cells, a period of time we had previously found to be required for OvCa cells to complete attachment to peritoneum and omentum (20). Untreated SKOV3ip1 cells attached to mouse peritoneum and omentum efficiently, while cells treated with the MMP-2/-9 inhibiting peptide showed a 56% decrease ($P < 0.005$) in binding as compared with the control peptide (Figure 2D). Together, these data indicated that either MMP-2 or MMP-9 mediated OvCa cell adhesion.

Initial attachment of OvCa cells to the abdominal cavity is mediated by MMP-2. The peptide used to inhibit adhesion in Figure 2 blocks both MMP-2 and MMP-9 activity (22, 23). Since MMP-2 and MMP-9 possess distinct regulatory capabilities and interact with a diverse spectrum of substrates, we used specific siRNAs and blocking antibodies to MMP-2 and MMP-9 to determine which of the type IV collagenases was required for adhesion. The selective siRNAs silenced both MMP-2 and MMP-9, as demonstrated by complete loss of MMP-2 and MMP-9 protein expression, while the scrambled siRNA had no effect (Figure 3A). To determine the effect of MMP-2 or MMP-9 inhibition on adhesion, SKOV3ip1 cells transfected with the siRNA or cells pretreated with the respective antibody against MMP-2 or MMP-9 were labeled fluorescently and then incubated with the 3D coculture (Figure 3B), full human omentum (Figure 3C), full human peritoneum (Figure 3D), or injected i.p. into nude mice (Figure 3E). Four hours later, adhesion was measured using a fluorescence reader that detects labeled cancer cells. Both pretreatment with the MMP-2 antibody and transfection with the MMP-2–specific siRNA significantly inhibited adhesion to the 4 different models ($P < 0.005$). This finding was not limited to SKOV3ip1 cells, since pretreatment of HeyA8, as well as several primary OvCa cells, with the MMP-2 antibody similarly inhibited adhesion to the 3D coculture, full human omentum, and peritoneum. Inhibition of MMP-9 had neither an effect on in vitro or on in vivo adhesion but did partially block invasion as previously reported (24) (Figure 3F). We ruled out the possibility that the effects identified for MMP-2 were instead mediated by MT1-MMP, since incubation of cells with a MT1-MMP neutralizing antibody (25) had no effect on OvCa cell adhesion (Figure 3F, left panel) but did block invasion (Figure 3F, right panel).

Host cells do not contribute the MMP-2 or MMP-9 necessary for peritoneal adhesion. Several studies have reported that MMP-2 and MMP-9 are expressed by activated stromal cells that thereby promote tumorigenesis (26). Moreover, in OvCa, expression of *MMP-2* and *MMP-9* mRNA has not only been localized to stromal cells but also to carcinoma cells (27). Therefore, we sought to determine the principal cellular source of MMP-2 and/or MMP-9 detected on OvCa cells. We found that blocking MMP-2 and MMP-9 function on HPMCs or HPFs with the MMP-2/-9 peptide or with the MMP-2 or MMP-9 antibodies did not alter adhesion of SKOV3ip1 cells (Figure 4A). Because other host-derived stromal and/or epithelial cells (e.g., adipocytes, vascular endothelial cells, leukocytes) not represented in the 3D culture might secrete MMP-2 or MMP-9 that could be used by the cancer cells in vivo, we took advantage of a genetic mouse model of *MMP2* and *MMP9* deficiency. Fluorescently labeled SKOV3ip1 cells were injected i.p. into *RAG-1*$^{-/-}$, *RAG-1*$^{-/-}$*/MMP2*$^{-/-}$, or *RAG-1*$^{-/-}$*/MMP9*$^{-/-}$ double-deficient mice (6, 28) and their adhesion to peritoneum was evaluated (Figure 4B). We found no difference in either peritoneal or omental attachment between SKOV3ip1 cells injected into any of the 3 experimental cohorts, indicating that MMP-2 and MMP-9 secreted

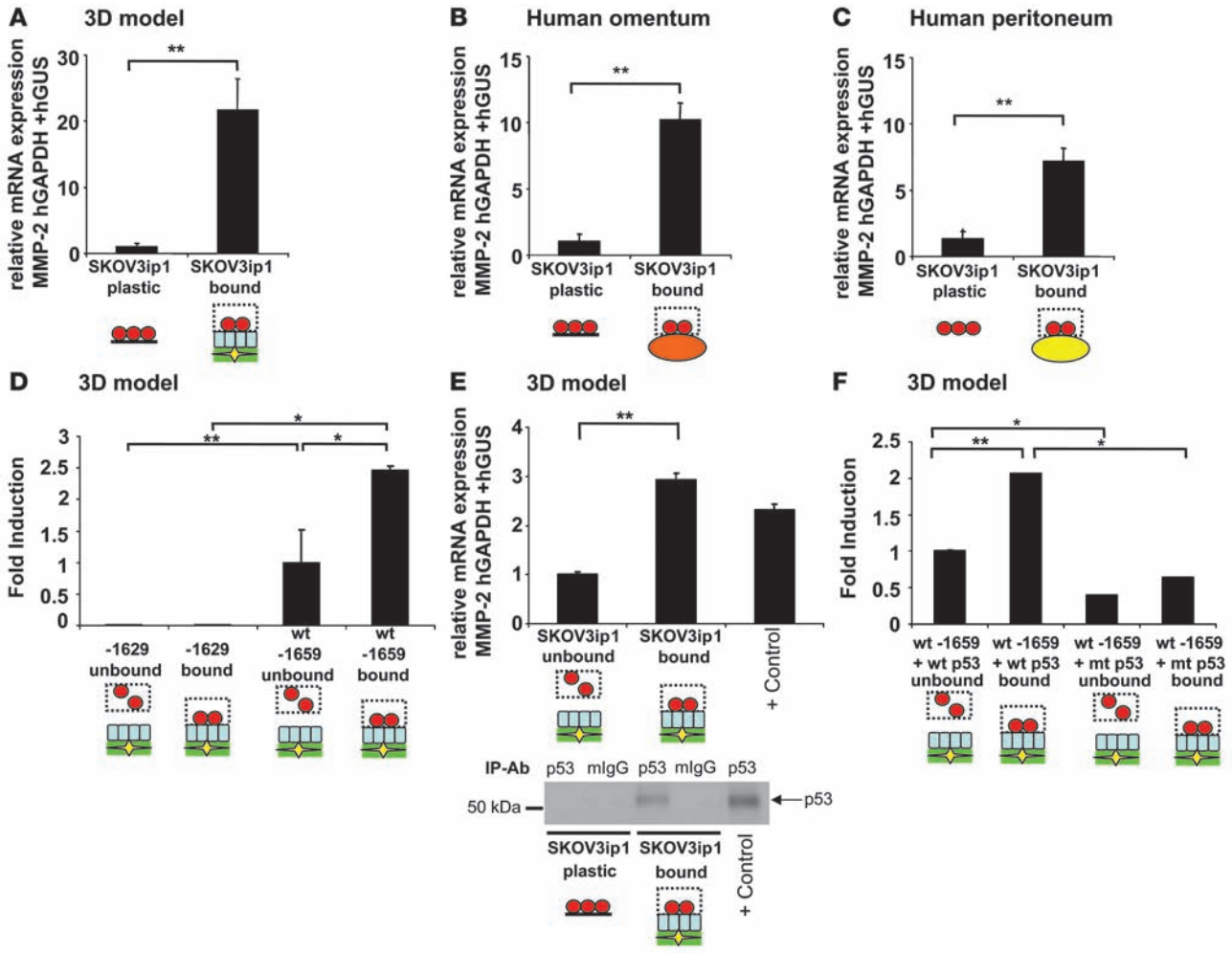

Figure 5

MMP-2 is transcriptionally upregulated in OvCa cells upon interaction with host stroma. SKOV3ip1 cells were added to the 3D culture (**A**), full human omentum (**B**), or full human peritoneum (**C**), and then cells were sorted by FACS. The relative expression of MMP-2 normalized to GAPDH, and huGUS was measured by TaqMan quantitative real-time RT-PCR. (**D**) SKOV3ip1 cells were transfected with 5 μg of the –1,659-bp (WT) or the –1,629-bp MMP-2 promoter and adhered to 3D model, and luciferase activity was measured. (**E**) The relative expression of p53 normalized to GAPDH, and huGUS was measured by TaqMan quantitative real-time RT-PCR (top panel). Protein lysates from SKOV3ip1 cells cultured on plastic or 3D culture (after sorting) were immunoprecipitated with control mouse IgG or monoclonal p53 antibody, and western blot analysis for p53 was conducted on lysates using a pantropic sheep anti-p53 antibody (bottom panel). Positive control was RKO mRNA or cell lysates. (**F**) SKOV3ip1 cells were cotransfected with 5 μg of the WT MMP-2 promoter and a WT or mutated p53 expression plasmid and adhered to the 3D model, and luciferase activity was measured. Luciferase activity was normalized to number of bound and unbound cells, and all assays were run in duplicate. **P* < 0.01, ***P* < 0.001. Each graph is representative of 3 independent experiments.

by host cells was not required for attachment of OvCa cells to abdominal peritoneum or omentum.

Adhesion induces MMP-2 transcription. In view of previous reports that MMP-2 is regulated transcriptionally (29, 30), we investigated whether interaction of OvCa cells with host cells stimulated *MMP-2* mRNA expression. Fluorescently labeled SKOV3ip1 cells were added to the various tissues, attached cells collected by fluorescent-activated cell sorting (FACS), and MMP-2 mRNA detected by quantitative real-time PCR. The relative expression of MMP-2 mRNA was 20-, 10-, and 7-fold higher in OvCa cells attached to the 3D coculture (Figure 5A), full human omentum (Figure 5B), or peritoneum (Figure 5C) than in OvCa cells adhering to plastic ($P < 0.00005$). This increase in MMP-2 mRNA led us to study whether adhesion induced MMP-2 promoter activity. In a systematic search, using sequentially deleted MMP-2 promoter fragments driving expression of a luciferase reporter construct, SKOV3ip1 cells were transfected with a series of 5′-promoter deletion constructs and added to the 3D culture for 4 hours to allow for adhesion (Figure 5D). We found that a sequence residing from –1,659 bp to –1,629 bp was both critical for induction of *MMP-2* promoter activation, following adhesion to the 3D culture, and for constitutive activity, indicating that *MMP-2* mRNA is transcriptionally upregulated in OvCa cells by interaction with host cells (Figure 5D). Indeed, within this 30-bp promoter region is a consensus p53 transcription factor binding site previously found to be important for constitutive activity of MMP-2 in HT-1080 cells (29). While

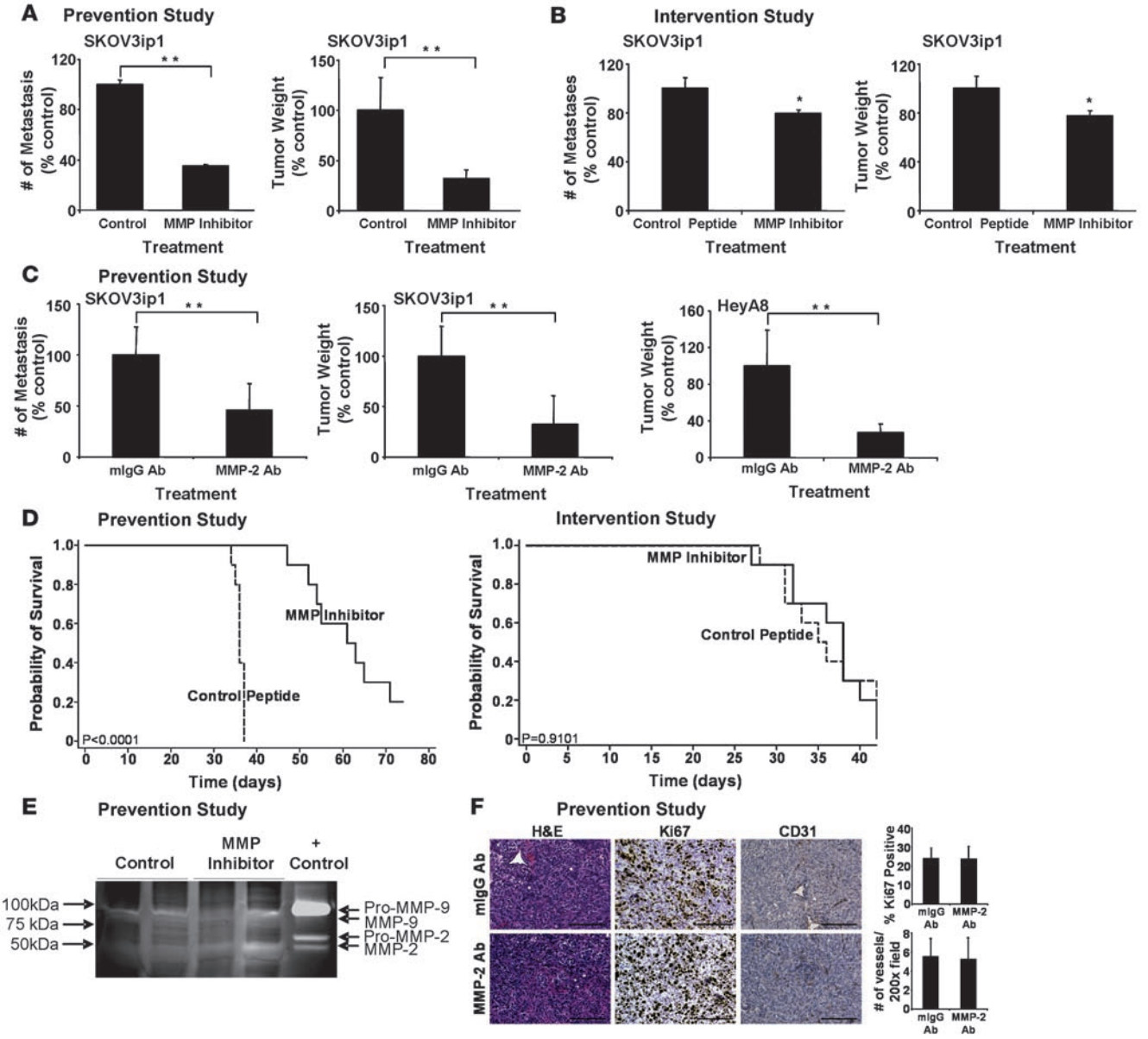

Figure 6

Single pretreatment of OvCa Cells with an MMP-2/-9 inhibitor or an MMP-2 antibody inhibits peritoneal metastases and increases survival. (**A**) Prevention study. SKOV3ip1 cells (1×10^6) were pretreated with MMPI or cyclic peptide and injected i.p. into nude mice. After 28 days, the number of metastasis and tumor weight were determined. $^{**}P < 0.001$. (**B**) Intervention study. SKOV3ip1 cells (1×10^6) were injected i.p. into nude mice. After 14 days of tumor growth, mice were treated with the MMPI 3 times per week for 3 weeks. After 28 days mice were sacrificed, the number of metastasis and tumor weight were determined. The columns represent the mean and the bars the SD. $^{*}P < 0.01$. (**C**) Prevention study. SKOV3ip1 cells or HeyA8 cells (1×10^6) were pretreated with an MMP-2 antibody or the isotypic specific control antibody and then injected i.p. into nude mice. After 28 days mice were sacrificed, the number of metastasis and tumor weight were determined. $^{**}P < 0.001$. (**D**) Survival in prevention and intervention studies. The treatment courses of the prevention and intervention studies were conducted as described above. Mice were sacrificed once they showed signs of distress, and Kaplan-Meier curves were calculated. (**E**) Tumor lysates from MMPI or control peptide treated mice were subjected to gelatin zymography as described in Figure 1B. (**F**) Sections of tumors from MMP-2 antibody or mIgG antibody–treated mice were stained with H&E, a Ki-67-specific antibody to detect proliferation, or a CD31-specific antibody to count microvessels. Scale bar: 100 μm.

there is evidence that SKOV3ip1 cells do not express p53 protein when cultured on plastic (31), our western blot and quantitative RT-PCR results showed that upon adhesion of cancer cells to the 3D culture, *TP53* mRNA and p53 protein expression was indeed upregulated (Figure 5E). Therefore, to determine if the p53 transcription factor binding site plays a functional role in the adhesion-mediated regulation of MMP-2, a mutated p53 expression construct (32) was cotransfected with the –1,659-bp MMP-2 promoter into SKOV3ip1, which was then plated on the 3D culture. Expression of mutant p53 inhibited adhesion-mediated induction

of the *MMP-2* promoter (Figure 5F), indicating that induction of *MMP-2* by adhesion is at least in part mediated through a p53 transcription factor binding site in the *MMP-2* promoter.

Single pretreatment of OvCa cells with an MMP-2/-9 inhibitor or an MMP-2 antibody prevents peritoneal metastases and increases survival. Our interpretation of the data resulting from the analyses above was that MMP-2 functionally regulates adhesion of OvCa cells to the mesothelium covering the abdominal cavity. Since adhesion is the first step in OvCa metastasis, we speculated that a 1-time pretreatment (prevention study) of OvCa with an MMPI might reduce peritoneal attachment and, therefore, diminish metastasis. Indeed, after 4 weeks, mice that received an i.p. injection of SKOV3ip1 that had been treated with an MMPI developed 68% fewer metastatic tumor nodules and 65% less tumor weight than mice injected with control treated cells ($P < 0.001$; Figure 6A). In contrast, mice were injected i.p. with SKOV3ip1 cells, and 2 weeks later, when their disease was established, they received repeated treatment for a total of 4 weeks (intervention study — a trial design that mimics treatment of advanced disease in humans). The mean number of metastases was reduced by only 20% and tumor weight by only 22% with the MMPI as compared with the control peptide ($P = 0.007$ and $P = 0.006$, respectively; Figure 6B). For both the tumor weight ($P = 0.0001$) and the total number of tumors ($P = 0.0001$), there was a statistically significant difference between preadministration of the inhibitor in the prevention study and repeated treatment after tumors had established in the intervention study.

Pretreatment of SKOV3ip1 with the MMP-2–blocking antibody also resulted in a significant reduction of metastatic nodules (54%; $P < 0.001$) and tumor weight (68%; $P < 0.001$) as compared with an isotypic control antibody (Figure 6C). Moreover, we found that these data were not limited to a single cell line, since pretreatment of HeyA8 cells with the MMP-2 antibody yielded similar results with a 72% ($P < 0.001$) reduction in tumor weight as compared with IgG treated controls (Figure 6C, right panel). Based on these findings, we then performed a survival study (Figure 6D), in which we found that a single pretreatment of SKOV3ip1 cells with the MMP inhibitory peptide increased median survival of inoculated mice to 63 days, whereas mice treated with the control peptide became distressed after an average of 36 days ($P < 0.0005$), a period comparable to the life span of untreated mice injected with SKOV3ip1 cells (33). In contrast, when established tumors (intervention study) were treated with the MMPI twice per week there was no statistically significant survival difference (Figure 6D).

We then analyzed tumors from the prevention studies to understand whether a single treatment with the MMPI or MMP-2 antibody affected other established MMP-2/-9 functions such as gelatinolytic activity, proliferation, apoptosis, or angiogenesis (Figure 6E). SKOV3ip1 tumors showed minimal gelatinolytic activity with no significant difference between MMPI or control treated cells (Figure 6E). The histologic appearance of tumors from the MMP-2 antibody prevention experiment was similar to that of tumors from control treated mice without evidence of necrosis or differences in apoptosis (Figure 6F and data not shown). There was also no difference in the percentage of Ki-67 positive cells in MMP-2 antibody (24% ± 7%) or mouse IgG antibody (24% ± 5.6%) treated tumors (Figure 6F) and no difference in microvessel density when staining with an antibody for CD31 (5 ± 2 versus 5 ± 2 no. of vessels per field; Figure 6F) or detecting VEGF by western blotting (data not shown). These results indicate that MMP-2 is involved in the earliest steps of metastasis, e.g., attachment, and that once a tumor is established, inhibition of MMP-2 does not affect processes involved in later stages of metastatic growth.

MMP-2 cleaves Vn and FN. MMP-2 can cleave various ECM proteins (3), raising the possibility that cleavage of 1 or more ECM components into smaller fragments allows for the improved adhesion of OvCa cells to ECM. To test this hypothesis, we first confirmed the presence of Vn and FN in human omentum. Vn and FN are highly expressed in the ECM of the human omentum, and mesothelial cells produce Vn and FN as shown by western blot analysis (Figure 7A). Vn is a known substrate for MMP-2 (3) and is known to promote cancer cell migration (34). However, FN has not been previously recognized as an MMP-2 substrate. To determine if MMP-2 cleaves FN, recombinant human MMP-2 was activated with p-aminophenylmercuric acetate (APMA), then incubated with full-length FN (Figure 7B), and resolved by SDS-PAGE. Silver staining of the gel revealed several known 120-kDa, 45-kDa, and 11-kDa FN fragments after incubation of FN with activated MMP-2 (Figure 7B), establishing FN as what we believe to be a novel MMP-2 target.

To test whether cleavage of Vn and FN by MMP-2 was of functional significance, adhesion assays were conducted on cleaved Vn or FN fragments. Indeed, significantly more SKOV3ip1 cells adhered to culture wells coated with MMP-2–cleaved Vn and FN than to wells coated with full-length Vn or FN alone (Vn, 1.4 ± 0.04-fold increase, $P < 0.0001$; FN, 1.7 ± 0.04-fold increase, $P < 0.0001$; Figure 7C), a finding which was also confirmed in HeyA8 cells (H.A. Kenny, unpublished observations). These results were validated in a nitrocellulose filter attachment assay (35), in which full-length or MMP-2–cleaved FN was resolved on a native Tris-HCl gel, transferred to nitrocellulose, and the binding of cells to filter detected by amido black cell staining (Figure 7D). OvCa cells attached to the 120- and 70-kDa MMP-2–cleaved fragment of FN, a finding that was consistent with fragments detected following MMP-2 cleavage (Figure 7B). Moreover, preincubation of cancer cells with MMP-2–cleaved Vn or FN competed with the adhesion of OvCa cells to the 3D culture (Vn, 1.3 ± 0.03-fold decrease, $P < 0.01$; FN, 1.4 ± 0.02-fold decrease, $P < 0.001$; Figure 7E). Taken together, these results indicate that MMP-2–cleaved FN and Vn promotes adhesion of OvCa cells.

Since OvCa cell adhesion is enhanced following cleavage of FN and Vn, we examined the functional contribution of the FN ($\alpha_5\beta_1$ integrin) and Vn receptors ($\alpha_V\beta_3$ integrin) during the initial phase of adhesion. α_5 and β_3 integrin were inhibited by siRNAs, and adhesion assays to the different ECM proteins performed (Figure 7F). Transfection with β_3 integrin siRNA significantly inhibited adhesion to MMP-2–cleaved Vn (1.7 ± 0.03-fold decrease; $P < 0.0001$) and FN (1.5 ± 0.04-fold decrease; $P < 0.0001$) fragments (Figure 7F). Moreover, transfection with α_5 integrin siRNA inhibited adhesion to MMP-2–cleaved FN fragments (2.7 ± 0.03-fold decrease; $P < 0.0001$). Lastly, we assessed the inhibitory function of α_5, β_1, and $\alpha_V\beta_3$ integrin–blocking antibodies (36) on OvCa cell adhesion and dependence on MMP-2 proteolytic activity. We first confirmed that treatment of OvCa cells with neutralizing antibodies against α_5, β_1, and $\alpha_V\beta_3$ integrin blocked OvCa cell adhesion (Figure 7G). Then, OvCa cells were pretreated with an antibody that inhibited MMP-2 proteolytic activity, followed by treatment with the respective integrin antibody. Indeed, blocking MMP-2 abolished the inhibitory function of the α_5, β_1, and $\alpha_V\beta_3$ integrin antibodies on OvCa cell adhesion. This effect was not observed when the MMP-2 antibody was substituted with isotype specific IgG and did not apply to all integrins, since the MMP-2 antibody could not abrogate the inhibitory function of a β_4 integrin–block-

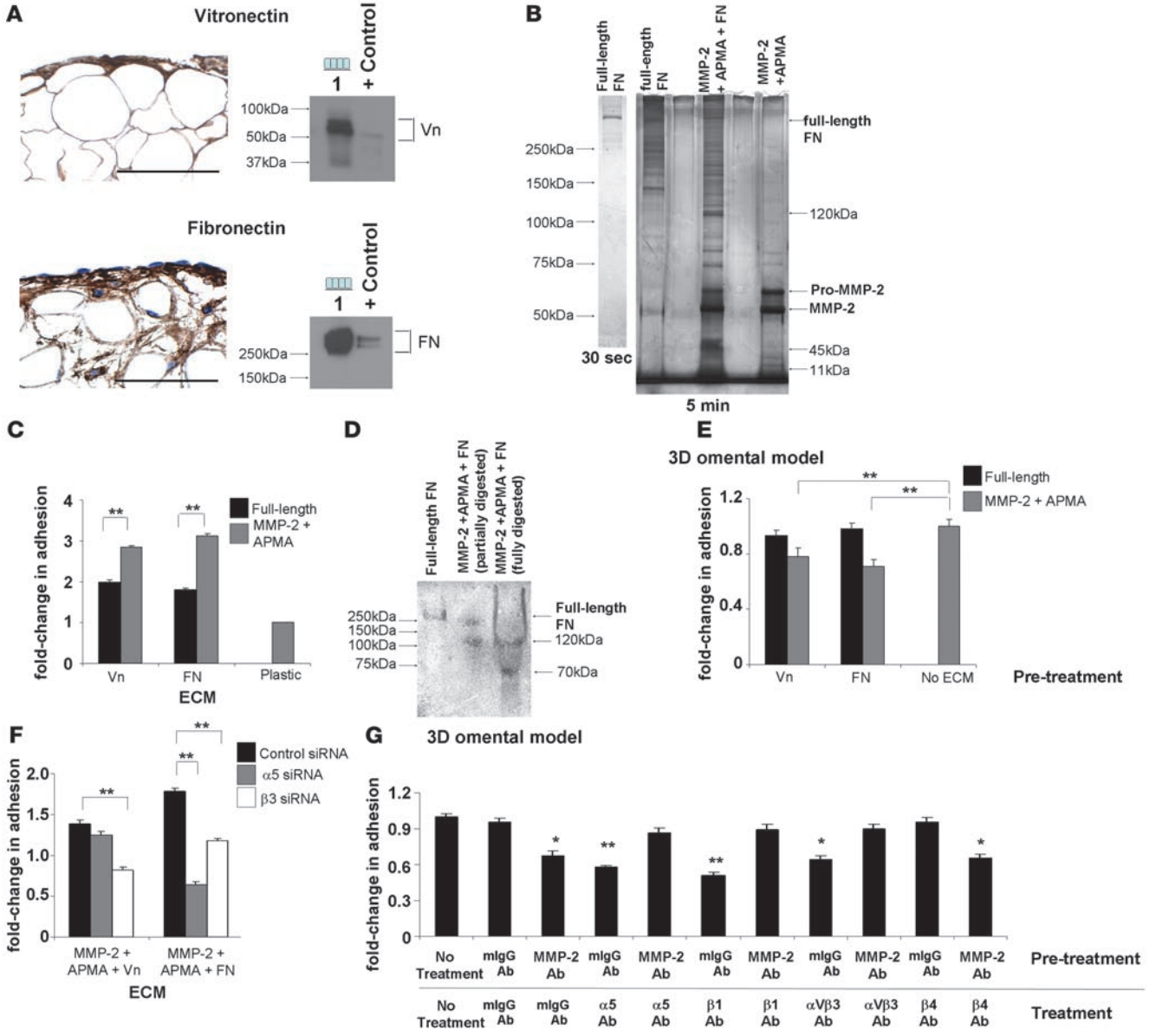

Figure 7

MMP-2 cleavage of Vn and FN increases OvCa adhesion. (**A**) Human omentum and peritoneum stained with Vn- or FN-specific antibodies (left panel). HPMCs were subjected to immunoblotting (right panel) using Vn- and FN-specific antibodies. Lane 1, HPMCs. HT-1080 CM was used as positive control. Original magnification, ×400. (**B**) Full-length FN was incubated with activated MMP-2, and fragments were resolved on a 10% Tris-HCl gel by silver stain analysis. (**C**) Adhesion assay of SKOV3ip1 cells to full-length and MMP-2–cleaved Vn or FN coated plates as described in Figure 2. (**D**) FN and MMP-2–cleaved FN were run on a native gel and transferred to nitrocellulose. An adhesion assay was performed with 1.0×10^7 SKOV3ip1 cells for 4 hours, the membrane was washed, fixed, and bound cells were stained. (**E**) Competition assays were conducted. SKOV3ip1 cells were preincubated with full-length Vn, MMP-2–cleaved Vn, full-length FN, or MMP-2–cleaved FN, and an adhesion assay to 3D coculture was conducted. (**F**) SKOV3ip1 cells were transfected with a siRNA specific for α_5 or β_3 integrin, and adhesion assay was performed to MMP-2–cleaved Vn or FN coated plates. (**G**) Fluorescently labeled SKOV3ip1 cells were pretreated with either an MMP-2 or a mouse isotype IgG antibody followed by treatment with α_5, β_1, $\alpha_v\beta_3$, β_4 integrin, or mouse isotype IgG antibodies. Subsequently, an adhesion assay was performed on the 3D model. $^*P < 0.01$, $^{**}P < 0.001$. Each graph and picture is representative of 3 independent experiments. Scale bar: 100 μm.

ing antibody. Taken together, these results indicate that MMP-2 enhances binding of cancer cells through $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin.

Discussion

Our contention that MMP-2 is a significant regulator of OvCa adhesion is supported by experiments demonstrating that a cyclic peptide preferentially inhibits MMP-2 gelatinolytic activity (22) and reduces adhesion to the 3D coculture, full human omentum/peritoneum, and in vivo binding to mouse omentum/peritoneum (Figure 2). These findings were confirmed with an MMP-2 selective siRNA and an MMP-2–blocking antibody (Figure 3). Pretreatment of OvCa cells with either the cyclic peptide or an MMP-2

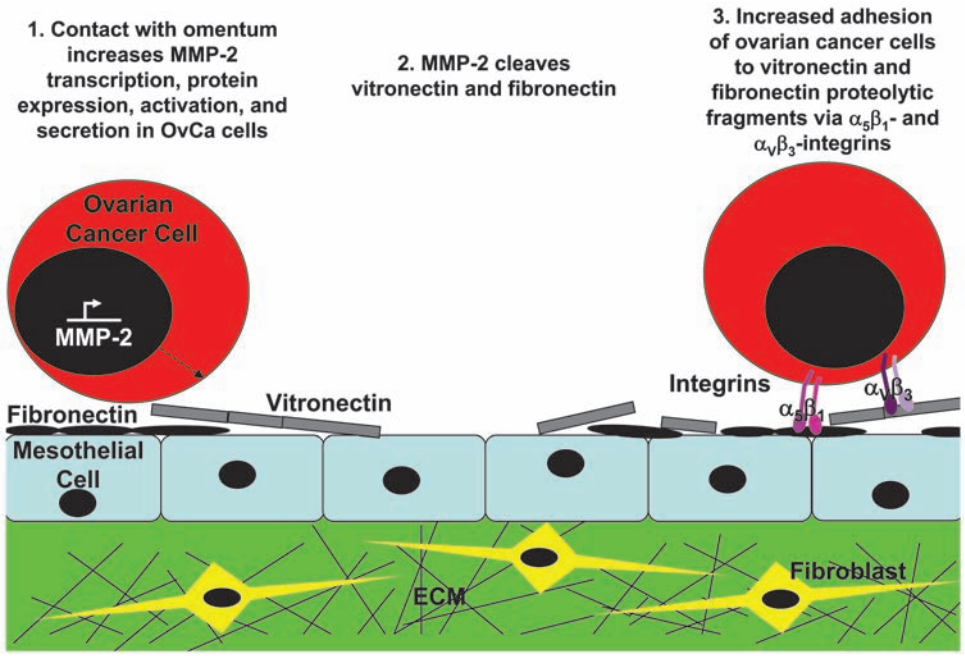

Figure 8
Proposed role of MMP-2 in early OvCa cell metastasis to the omentum and peritoneum.

neutralizing antibody abrogated cancer cell adhesion, reduced the number of metastases and tumor load, and prolonged survival in xenograft models. The enhanced efficacy of the MMPI when delivered early, prior to secondary tumor development, was not limited to OvCa. In a T cell lymphoma mouse model, treatment with a selective gelatinase inhibitor reduced liver metastasis and increased survival when the inhibitor was preincubated with the cancer cells prior to tail-vein injection but not when treatment was initiated 1 day following tumor cell inoculation (37).

We found that induction of MMP-2 in OvCa cells by normal omental fibroblasts and mesothelial cells involves direct cell-cell interaction, extending previous reports that dermal (21) and ovarian (38) fibroblasts induce MMP-2 expression in cancer cells. MMP-2 is not provided by the host, since deletion of host-derived MMP-2 or MMP-9, using RAG1/MMP-2 and MMP-9–deficient mice, did not alter OvCa cell adhesion. The specific interaction between mesothelial cells/fibroblasts and OvCa cells may explain why OvCa metastasis favors the peritoneum and omentum and rarely seeds to secondary sites that are not covered by mesothelium. In our studies, MMP-2 was induced in the cancer cells upon binding to mesothelium. Still, in situ hybridization in different cancers, including ovarian (27, 39), has localized MMP-2 mRNA expression to fibroblasts. However, these represent a snapshot of MMP expression at an advanced tumor stage, when the OvCa has already established. In contrast, our study examines the initial step of OvCa metastasis, when cancer cells "meet" unprepared, resting mesothelial cells and fibroblasts. In the earliest phase of metastasis, contact of mesothelium with OvCa cells induces MMP-2 mRNA expression in the cancer cells through a consensus p53 transcription factor binding site in the MMP-2 promoter at –1,659 bp. Consistent with this finding, we found that upon attachment of SKOV3ip1 cells to the 3D culture, p53 protein and mRNA expression is induced and mutation of the p53 binding site in the MMP-2 promoter abrogates induction of MMP-2.

The SKOV3 cell line is widely used as a p53-null cell line, and previously no p53 mRNA or protein was detected in SKOV3 or SKOV3ip1 cells when cultured on plastic (31, 40, 41). However, only 1 study investigated the status of p53 alleles using Southern blot analysis, wherein a deletion or rearrangement of only 1 of the p53 alleles was reported (42). On the contrary, SKOV3 cells were reported as wild type for p53 in another study (43). Given that the SKOV3ip1 cell clone we used also does not express p53 on plastic but that p53 mRNA and protein are induced in the SKOV3ip1 cells upon binding to the 3D culture, we suggest that in the SKOV3ip1 cells one p53 allele is still intact (as suggested by the Southern blot in ref. 42), allowing for inducible p53 expression.

The mechanism by which MMP-2 enhances peritoneal adhesion of OvCa cells involves enzymatic activity and cleavage of FN and Vn. When a panel of integrin antibodies was tested for their ability to inhibit adhesion, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrin–blocking antibodies inhibited adhesion, but this effect was abolished when OvCa cells were preincubated with the MMPI or an MMP-2 antibody. This finding led us to investigate the possibility that MMP-2 cleaves Vn and FN that is highly expressed in peritoneal and omental basement membranes, as well as on HPMCs. Indeed, we found that MMP-2 cleaves FN and Vn. In addition, OvCa cells adhere more efficiently to the smaller FN and Vn cleavage products than the full-length proteins, and the increased adhesion is abrogated when $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrin are knocked down by siRNA. We propose the following general mechanism for the initial steps of OvCa adhesion. Contact of OvCa cells with mesothelium induces *MMP-2* transcription, followed by protein expression and activation. MMP-2 then cleaves various ECM components, including Vn and FN, into smaller fragments, thereby allowing for improved binding of OvCa adhesion receptors ($\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrin) to fragmented ECM (Figure 8). This model is consistent with a report demonstrating that specific cleavage of laminin-5 by MMP-2 can induce migration of breast epithelial cells (44), and that $\alpha3\beta1$ integrin on sarcoma cells selectively binds laminin-5 within exposed regions of lung vasculature (45).

We found that MMP-9 is highly expressed by mesothelial cells and fibroblasts and that OvCa cells express MMP-9 once they bind the 3D coculture. Nevertheless, we concluded that MMP-9 expression was not significant for initial adhesion, since we saw no inhibition of adhesion when blocking MMP-9. Nonetheless, several reports suggest that MMP-9 promotes growth and angiogenesis. Huang et al. (46) identified tumor-infiltrating macrophages as a significant source of MMP-9 and demonstrated that SKOV3ip1 cells growing in MMP-9–deficient mice are less metastatic. In a skin carcinogenesis model, MMP-9 supplied by innate immune cells is critical for tumor progression (26), tumor cell proliferation, and angiogenesis

(47). In the RIP1-Tag2 model of pancreatic islet carcinogenesis, MMP-2 was important for tumor initiation but played no role in angiogenesis (7). These findings, together with our studies, assign unique functions to MMP-2 and MMP-9 during tumorigenesis. MMP-2 is important for adhesion, while MMP-9 exerts a functional role during angiogenesis and tumor progression.

While we remain disappointed by the failure of MMPI in clinical studies aimed at targeting late-stage disease, we are optimistic that our findings, which demonstrate that inhibition of MMP-2 during early metastasis provides a survival advantage, will encourage the reassessment of MMPI as an anticancer therapeutic agent. The MMPI, marimastat, that has selective affinity for MMP-2/-9, was evaluated in a phase III trial enrolling patients with platinum resistant, recurrent OvCa. Although the trial was not formally published, abstracts and reviews (11) reported that treatment with marimastat did not block tumor growth or improve survival, a result that is in line with other unsuccessful trials of MMPI in cancer (8, 10). The data presented here, demonstrating that MMP-2 is important for adhesion and early metastasis, explain at least partially, why marimastat failed in the treatment of OvCa — patients treated with marimastat had widely metastatic tumors that are less dependant on MMPs for continued growth and survival. Our prevention study shows clearly that treatment with an MMP-2 inhibitor will only exhibit efficacy if administered prior to ovarian tumor cell attachment to the peritoneal cavity. While the clinical relevance of beginning therapy before tumor inoculation is at first glance debatable, there is a subgroup of OvCa patients who might benefit from early treatment with an MMPI. These are patients whose disease at the time of surgery is limited to the ovary but have tumor cell positive ascites (FIGO stage IC to IIIA), or patients who at the end of surgery were rendered macroscopically tumor free and have only presumed "microscopic disease". Early i.p. treatment with an MMPI may reduce peritoneal attachment, although it would have less impact on the growth of cells that have already attached and have formed colonies. In summary, we have demonstrated that adhesion of OvCa cells to the peritoneal cavity is mediated, at least in part, by MMP-2 through cleavage of FN and Vn and that in a mouse model, pretreatment with an MMPI can reduce metastasis as well as significantly prolong survival.

Methods

Reagents. Collagen I (rat tail), FN, and Vn were purchased from BD Biosciences. Anti-vimentin, anti–prolyl-hydroxylase, anti-Ki67, and anti-CD31 antibodies were purchased from Dako Cytomation. Anti-CAM 5.2 against cytokeratin 8 was from Becton Dickinson. The β_1 integrin antibody was purchased from Santa Cruz, while MT1-MMP (clone AB8102), TIMP-2, β_4 integrin (clone ASC-3), $\alpha_v\beta_3$ integrin (clone LM609), α_5 integrin (clone P1D6), and MMP-2 (clone CA-4001) antibodies were purchased from Chemicon. Anti-actin antibody was from Cell Signaling Technology. The human OvCa cell lines, SKOV3ip1 and HeyA8, were provided by Gordon B. Mills (MD Anderson Cancer Center, Houston, Texas, USA). Anti–MMP-2 and –MMP-9 (clone IM09L) monoclonal antibodies were purchased from EMD Bioscience. MMP-2/MMP-9 Inhibitor III, monoclonal anti-p53 (clone Ab-5) antibody, pantropic anti-p53 (clone Ab-7) antibody, fatty-acid free bovine serum albumin, and streptavidin-horseradish peroxidase conjugate were purchased from Calbiochem. Negative control, MMP-2, MMP-9, and α_5 and β_3 integrin siRNA were purchased from Ambion Inc.

Animal husbandry. Mice carrying homozygous-null mutations in the *MMP2* (28), *MMP9* (48) and *RAG-1* gene (6) were backcrossed into the FVB/n mouse strain and maintained in the homozygous-null (–/–) state. Immunodeficient *MMP2*$^{-/-}$ and *MMP9*$^{-/-}$ mice were generated by successive intercrossing (49). For all other experiments female athymic nude mice were used. All procedures involving animals were approved by the Institutional Committee on Animal Care, University of Chicago.

Adhesion and invasion assay. Primary OvCa cells were isolated from ascites as described (50). HPMCs and HPFs were isolated from normal human omentum, and purification was verified by vimentin, keratin 8 (CAM5.2), and prolyl-hydroxylase stainings (20). HPMCs and HPFs at early passages (passages 1–3) were used for the experiments to minimize dedifferentiation and modification of the original phenotype.

The 3D culture was assembled by embedding HPFs in collagen I and plating HPMCs as a confluent layer on top (20). Adhesion assays to the 3D culture and full human omentum or peritoneum were conducted as previously described (20). Each adhesion assays was run in triplicate. For adhesion assays to mouse peritoneum, SKOV3ip1 cells were fluorescently labeled with CMFDA (Invitrogen), and a single-cell suspension (4×10^6 cells/ml in 0.5 ml PBS) was injected into the peritoneal cavity of athymic nude or RAG1$^{-/-}$, MMP2$^{-/-}$, or MMP-9$^{-/-}$ mice. After 4 hours, mice were sacrificed and full-thickness peritoneum and omentum excised. After washing to remove nonadherent cells, the tissue was lysed with 1% NP-40 and fluorescence was measured with a fluorescence spectrophotometer (Synergy HT) (33).

For the nitrocellulose adhesion assay (35), FN digested with APMA-activated MMP-2 or full-length FN was resolved on a native Tris-HCl gel (4%–20%). Proteins were transferred on nitrocellulose, the blot was washed with PBS and incubated with SKOV3ip1 cells for 4 hours. After adhesion, the blot was washed, cells were fixed with 10% formalin, stained with 0.1% amido black, and destained with a methanol/acetic acid/water solution (90:2:8). Cells bound to nitrocellulose stain dark blue. For the competition adhesion assays, SKOV3ip1 cells were preincubated with FN or cleaved FN fragments, and then an adhesion assay performed. The invasion assay was conducted for 24 hours as described (33).

Inhibition experiments. The SKOV3ip1 cells were incubated for 18 hours with MMP-2/-9 inhibitor (10 μm), anti–MMP-2 (4 μg/ml), anti–MMP-9 (6 μg/ml), or anti–MT1-MMP (12 μg/ml) neutralizing antibody. For integrin experiments, the cells were pretreated with anti–MMP-2 or mouse IgG antibody (18 hours), followed by treatment with antibodies against α_5, β_1, β_4, $\alpha_v\beta_3$ integrin or specific isotype mouse IgG (10 μg/ml). The cells were then used for in vitro or in vivo adhesion assays. MMP-2, MMP-9, α_5, β_3 integrin, and control siRNA constructs were transfected into 2.4×10^5 SKOV3ip1 cells using siPORT NeoFX transfection reagent (Ambion Inc.). After culturing cells for 72 hours (optimal knockdown time), they were used in the in vitro or in vivo adhesion assays.

MMP activity measurement. Gelatinolytic zymography was performed as described with conditioned serum-free media (51).

Cell-surface–associated MMP activity was measured using a fluorogenic peptide (DQ gelatin; Invitrogen). A total of 25,000 cells were plated in a 96-well plate. The plates were incubated at 37°C and fluorescence was measured. Samples were run in quintuplet.

Western blot analysis. After adhesion of CMFDA-labeled SKOV3ip1 cells to the 3D culture, cells were sorted by FACS on a MoFlo (Dako Cytomation). This procedure separated labeled SKOV3ip1 from fibroblasts and mesothelial cells after their coculture (Figure 1D). Sorted cells were pelleted and lysed in ice-cold RIPA buffer. An equal amount (15 μg) of cell extracts were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and blocked with NET-gelatin (52). Membranes were incubated with mouse anti–MMP-2 (1:1,500), mouse anti–MMP-9 (1:1,500), rabbit anti–MT1-MMP (1:1,000), mouse anti–TIMP-2 (1:500), mouse anti-actin (1:50,000), or mouse anti-FN antibody (1:2,000; soluble IST-3) overnight at 4°C. The blots were then incubated with secondary horseradish peroxidase-conjugated IgG or IgM and visualized with enhanced chemiluminescence reagents.

Quantitative real-time RT-PCR. After adhesion of CMFDA-labeled SKOV-3ip1 cells to the 3D culture, cells were sorted by FACS in PBS. cDNA was synthesized after total RNA extraction using the Applied Biosystems cDNA archive kit. After RT, real-time PCR was performed using a Prism 7500 (Applied Biosystems), with predesigned and validated TaqMan probes for MMP-2, p53, GAPDH, and huGUS. The relative mRNA expression of MMP-2 or p53 was normalized to GAPDH and huGUS mRNA expression (Applied Biosystems). The reactions were run in triplicate using the following conditions: 1 cycle at 50°C for 2 minutes, followed by 10 minutes activation of the polymerase at 95°C. Subsequently, 40 cycles were performed at 95°C for 15 seconds and 60°C for 60 seconds. The median Ct value was determined, and data was expressed as fold change of relative mRNA expression using the comparative Ct method (53).

Transfections. SKOV3ip1 cells were transiently transfected with the full-length MMP-2 promoter (–1,659 bp or 5′-deletions (30) using SuperFect Transfection Reagent (QIAGEN). Eighteen hours after transfection, cells were trypsinized for adhesion assays, and the SKOV3ip1 cells (1.5×10^6 /well) were added on the 3D culture. After 4 hours cells were detached, lysed, and luciferase activity was analyzed (54).

Immunoprecipitation. Immunoprecipitation western blot analysis was performed on SKOV3ip1 cells that were bound to plastic or bound to 3D culture after FACs sorting as described (55).

Treatment studies. For the intervention study, SKOV3ip1 cells (1×10^6) were injected into the peritoneal cavity of nude mice. Fourteen days after injection, the control peptide (200 μg/injection/mouse) or MMPI (200 μg) was injected twice per week for 3 weeks (10 mice/group). For the prevention study, SKOV3ip1 cells (1×10^6), pretreated with control (PBS alone) or MMPI (10 μM), were injected i.p., and 28 days after injection the mice were sacrificed (10 mice/group). The number of tumor colonies was counted and the tumor was weighed (33). For the prevention survival study, SKOV3ip1 cells (1×10^6), pretreated for 18 hours with control peptide (10 μm) or MMPI (10 μm), were injected i.p. into female athymic nude mice (10 mice/group), and animals were sacrificed at the first signs of distress per institutional guidelines. For the intervention survival study, treatment was started after 14 days and continued 2 times per week for 3 weeks (10 mice/group).

Immunohistochemistry. Formalin-fixed tumors were stained with H&E, or immunohistochemistry was performed with antibodies against Ki67 (1:100), CD31 (1:50), and FN (soluble IST-3) (Sigma-Aldrich).

MMP-2 cleavage of Vn and FN. Recombinant human MMP-2 (a kind gift of William Stetler-Stevenson, National Cancer Institute [NCI], Bethesda, Maryland, USA) was activated with APMA for 1 hour at 37°C. Full-length Vn or FN was cleaved by activated MMP-2 for 2 hours at 37°C. One hundred nanograms of MMP-2 and APMA-activated MMP-2 were analyzed by gelatin zymography. MMP-2 cleavage of FN were analyzed on a 10% Tris-HCl gel and silver stained.

Statistics. Adhesion assays were performed in triplicate, and at least 3 independent experiments were conducted. Gelatinase assays were performed in quintuplet, and at least 3 independent experiments were conducted. The mean ± SD are reported. Significant changes were determined by 2-sided, unpaired *t* tests. Kaplan-Meier survival estimates were calculated to determine significant changes in prevention survival study.

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Immune cells as mediators of solid tumor metastasis

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Abstract Outgrowths of disseminated metastases remain the primary cause of mortality in cancer patients; however, molecular and cellular mechanisms regulating metastatic spread remain largely elusive. Recent insights into these mechanisms have refined the seed and soil hypothesis and it is now recognized that metastasis of solid tumors requires collaborative interactions between malignant cells and a diverse assortment of "activated" stromal cells at both primary and secondary tumor locations. Specifically, persistent pro-tumor immune responses (inflammation), now generally accepted as potentiating primary tumor development, are also being recognized as mediators of cancer metastasis. Thus, novel anti-cancer therapeutic strategies targeting molecular and/or cellular mechanisms regulating these collaborative interactions may provide efficacious relief for metastatic disease. This review focuses on recent literature revealing new mechanisms whereby immune cells regulate metastatic progression, with a primary focus on breast cancer.

Keywords Metastasis · Breast cancer · Inflammation

1 The problem of metastasis

Metastatic tumors represent the greatest threat to cancer patient mortality. For example, when breast cancer is diagnosed early and metastases are not present, 5-year survival is >88%; however, if metastases are also present, long-term survival is significantly diminished (~10%) [1], implying that therapeutic strategies targeting cellular and/or molecular mechanisms regulating rate-limiting steps in metastatic dissemination may reduce cancer mortality.

1.1 Collaboration of seed and soil

In the late nineteenth century, Paget and colleagues provided evidence that metastatic spread of tumors was not governed by random events but rather by regulated processes [2]. Paget assessed 735 breast cancer case histories and recognized that metastases were preferentially found in liver, as compared to other organs with similar blood flow, such as spleen [2]. This fundamental observation led to the "seed and soil" hypothesis stating that tumor cells (seeds) preferentially metastasize to discrete locations (soils). In more recent history, molecular mechanisms regulating dissemination of tumor cells and their selective outgrowth at ectopic sites has been the subject of great investigation. What is now clear is that metastasis is regulated not only by intrinsic genetic changes in malignant cells, but also by context of the microenvironment (Fig. 1). Thus, while genetic and epigenetic changes in neoplastic cells are likely 'initiators' of carcinogenesis, several lines of evidence indicate that stromal cell responses in neoplastic tissues 'promote' and/or regulate progression to malignancy and/or subsequent metastatic spread. Stromal cells contributing to these events include (myo)fibroblasts, vascular cells, infiltrating leukocytes and specialized mesenchymal support cells unique to each organ microenvironment. Of these cellular components, a growing body of research has implicated tumor-infiltrating leukocytes as causal players in primary cancer development, progression and metastatic dissemination [3–9]. This review highlights recent insights

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into how interactions between neoplastic epithelial cells and activated leukocytes regulate hematogenous spread of tumors by fundamentally altering the behavior and malignant potential at both the primary site and the secondary metastatic niche.

1.2 Metastasis is a multi-step process

Hematogenous spread of malignant cells consists of a series of steps all of which are thought prerequisite for metastatic outgrowth in distal organs [10–13] (Fig. 1). Generally, these steps include local invasion towards and entry into blood vasculature (intravasation), survival in circulation, arrest in distant capillary beds and/or "homing" to distal organs, exit from blood vasculature (either by extravasation or vascular breach), and eventual outgrowth and re-establishment of malignant growths in secondary locations [10, 14] (Fig. 1). While it has been recognized for many years that movement of neoplastic cells is not a random process, molecular and cellular mechanisms governing their movement and survival through foreign tissue environments, and parameters modulating their residence at ectopic sites have remained uncertain. Several contrasting theories have emerged to explain metastatic specificity. The 'homing' theory suggests that organs distal to sites of primary malignancy actively attract and/or arrest ('trap') malignant cells via expression of adhesion receptors, e.g., selectins, or secretion of soluble chemotactic factors, e.g., chemokines. Indeed, there is good experimental evidence implicating chemokine receptors and their ligands for the chemoattraction aspect of the 'homing' theory [15,16]. In addition, identification of 'molecular addresses' or adhesion receptors on endothelial cells in vascular beds of distal organs that specifically 'trap' circulating malignant cells supports the active "arrest" view of homing [17, 18].

In contrast, the 'fertile soil' theory proposes that different organ environments provide optimal growth conditions for specific circulating cell types [2]. Since underlying mechanisms supporting these theories are not mutually exclusive, it seems likely that tissue-specific and cancer-specific mechanisms/molecules likely govern a malignant cells journey to and survival in ectopic tissue. That said, chronically-activated immune cells (inflammation) have emerged as functionally significant regulators of primary cancer development as well as survival and proliferation in ectopic tissues.

2 Inflammation as a regulator of cancer development and metastasis

The presence and significance of leukocytic infiltrates in developing neoplasms is now undisputed [3, 5, 7–9, 19].

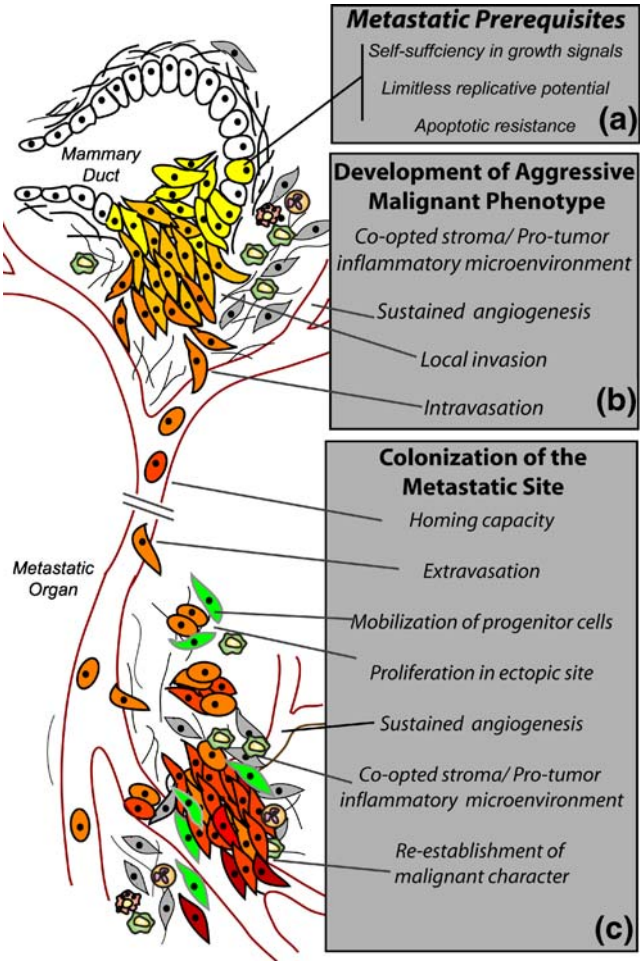

Fig. 1 Pro-tumor inflammation potentiates metastatic progression. Pro-tumor inflammatory responses potentiate malignancy and hematogenous metastatic spread process by promoting critical steps in the metastatic process. Productive metastases require, (**a**) oncogenic transformation and genomic instability in *initiated* cells, resistance to apoptotic and cell death programs, self-sufficiency for growth signaling and limitless replicative potential. (**b**) Interactions between neoplastic cells and pro-tumor pro-inflammatory microenvironments facilitate malignant conversion and favors selection for malignant cells with metastatic potential by engendering development of an aggressive malignant phenotype characterized by sustained angiogenesis, local invasion and vascular intravasation by neoplastic cells. (**c**) Pro-tumor inflammatory microenvironments support colonization of malignant cells in metastatic sites by enhancing neoplastic cell homing to target metastatic organs, extravasation out of vasculature, mobilization of bone marrow-derived progenitor cells, activation and sustained angiogenesis at secondary site, and enhanced neoplastic cell proliferation and survival in the ectopic site

Initially it was believed that leukocytic infiltrates in developing tumors were an attempt by the host to eradicate malignant cells. While, some leukocytes certainly have this potential, i.e., cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells [9, 20], other leukocytic cell types, most notably innate immune cells, i.e., mast cells (MCs), immature myeloid cells, granulocytes, and macrophages, instead potentiate tumor progression [5, 8, 9, 21,

22], and enhance neoplastic cell survival. Upon entry into the neoplastic microenvironment, infiltrating leukocytes become alternatively activated and manifest a pro-tumor phenotype as defined by activation of cellular programs involved in immune tolerance and tissue remodeling [22, 23]. During premalignant progression, a consequence of alternative activation of leukocytes is promotion and elaboration of a microenvironment rich in extracellular matrix (ECM) remodeling proteases, and increased presence of pro-survival, pro-growth and pro-angiogenic factors that further enhance proliferative and invasive capacities of neoplastic cells [8]. Such pro-tumor inflammatory microenvironments promote not only malignant conversion and development of solid tumors, but also dissemination of neoplastic cells into blood vasculature by driving invasive capacity of malignant cells [24, 25], expansion of angiogenic vasculature [26], and neoplastic cell entry into blood vessels (and lymphatics) [27].

2.1 Immune-regulation of angiogenic programs

The potential of neoplastic cells to spread locally (i.e. progress to malignancy) and systemically (i.e. metastasize) is linked to activation of angiogenic vasculature [14]. Tumor-associated blood vessels generated by angiogenesis support tumor growth and development, as well as provide escape routes for malignant cells to intravasate into the circulation [14, 27]. While several studies have found that some highly metastatic breast cancer cell lines have upregulated expression of genes encoding pro-angiogenic factors such as vascular endothelial growth factor (VEGF) that favor activation of angiogenic vessels [28], tumor angiogenesis is also regulated by activated immune cells attracted to neoplastic microenvironments [29, 30, 22].

In breast and ovarian cancer, tumor-associated macrophages (TAMs) represent one such pro-angiogenic collaborator [31, 32]. In breast carcinomas, macrophages are one of the most abundant innate immune cell types and TAMs have been found to enhance angiogenic programming by production of pro-angiogenic factors such as VEGF and proteases, i.e., urokinase-type plasminogen activator (uPA) and matrix metalloproteinase (MMP)-9 [31]. Using a mouse model of aggressive metastatic mammary carcinogenesis (MMTV-PymT mice) [33], studies by Pollard and colleagues revealed that deletion of colony stimulating factor-1 (CSF-1) resulted in attenuated angiogenic responses [31], reduced malignant progression and inhibition of pulmonary metastasis due to failed macrophage recruitment into the neoplastic microenvironment [34, 35]. Furthermore, intravital imaging of murine mammary carcinoma models revealed that macrophages affect metastasis by regulating motility of tumor cells away from the primary mass and by regulating extravasation of tumor cells from peripheral blood into tissue [24, 25]. In accordance with these observations, the number of macrophages present in tumor stroma correlates with increased microvessel density, tumor size, cell proliferation in breast cancers and decreased survival in breast and endometrial cancers [36–40]. In addition, Oosterling et al. found a link between presence of macrophages in metastatic sites and growth of metastatic tumors [41]. When macrophages were selectively depleted in the peritoneal cavity or liver, CC531 colon tumor cell lines injected into either the peritoneum or portal vein of liver formed tumors more slowly compared to control mice [41]. Taken together these data demonstrate involvement of CSF-1 and EGF in macrophage-mediated effects on metastasis formation. However, it is likely that other macrophage-derived factors, in addition to EGF, are also involved in metastasis formation. Taken together these experimental and clinical studies highlight the significance of immune cell infiltrates in neoplastic stroma, whose presence potentates metastatic spread by activating angiogenic vasculature [31, 35].

In addition to macrophages, other leukocytes present in neoplastic stroma also regulate angiogenesis. MCs accumulate during premalignancy and at the periphery of solid tumors, and have been found to exert potent pro-angiogenic/pro-tumoral effects [30, 42–44]. MCs can directly promote angiogenesis by their production of proteases, particularly MMP-9, or angiogenic growth factors such as platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and VEGF, as well as production of diverse mediators such as heparin and interleukins (IL)-6 and -8. In addition, MCs indirectly stimulate angiogenesis by secreting serine proteases (chymases and tryptases) that can activate pro-MMPs and stimulate fibroblast proliferation and fibrillar collagen synthesis [21, 30, 43, 44]. Recruitment of other immune cell types, including immature myeloid cells, granulocytes, and Tie2-expressing monocytes, may also play a role in regulating tumor-associated angiogenesis by their production of canonical proangiogenic factors such as VEGF, IL-8, MMPs and elastases [45–48]. Taken together, immune cell infiltrates in neoplastic stoma promote malignant progression and conversion, in part by enhancing angiogenic programs and in so doing facilitating dissemination of malignant cells into blood vasculature. Though promotion of angiogenesis is a prerequisite for neoplastic cell dissemination into blood vasculature, activation of angiogenesis is not in itself sufficient to elaborate a metastatic phenotype.

2.2 Immune regulation of tissue remodeling and intravasation

In addition to promoting angiogenesis, inflammatory infiltrates promote metastatic dissemination by enhancing

migratory/invasive potential of neoplastic cells through production of tissue remodeling proteases, cytokines and growth factors [8, 22]. MCs and macrophages provide a wide range of proteases, including serine, cysteine and metallo proteases [22, 49–51]. These leukocyte-derived proteases facilitate malignant progression by remodeling the structural components of ECM, such as fibrillar collagens, elastins or fibrin, that in-turn can provide both conduits for malignant cell egress, as well as generating ECM fragments with diverse bioactivities. For example, the proteolytic activities of MMP-2 expressed by macrophages and other leukocytes has been found to regulate release of cryptic fragments by cleaving laminin-5 γ2 chains that in-turn mimic EGF receptor (EGFR) ligands and induce cell motility and invasion in EGFR expressing breast carcinoma cell lines [52, 53]. In addition to generating bioactive ECM molecules, leukocyte-derived proteases also trigger release of growth and angiogenic factors sequestered in neoplastic stroma, as well as processing of cell–cell and cell–matrix adhesion molecules [54, 55]. Leukocyte derived MMP-7 has been found to process pro-heparin-bound-EGF (HB-EGF) into its' bioactive form [56] and a recent study by Wang and colleagues demonstrated that enhanced bioactivity of HB-EGF resulted in repression of the adherence junction protein E-cadherin in pancreatic carcinoma cells, and thus potentiated their invasive capacities [57]. In addition, leukocyte-derived MMP-7 and cathepsins (e.g. cathepsin B) can further facilitate tumor cell motility and invasion by directly cleaving E-cadherin [58, 59]. Thus, leukocyte-derived ECM remodeling proteases have the capacity to alter neoplastic cell adhesion and motility and facilitate neoplastic cell movement into and through stroma.

Histological analysis of breast carcinomas has demonstrated that leukocytic infiltrates (including macrophages) are coordinately present at the site and time of basement membrane breach and tumor cell egress [31, 60]. Taken together these studies indicate that inflammatory infiltrates initiate a cascade of peri-cellular proteolytic activities regulating remodeling of ECM and basement membrane components, and generation of concentration gradients of pro-tumor factors that promote neoplastic cell invasion through stroma towards tumor-associated vasculature.

The contribution of growth factors, chemokines and morphogenic factors that stimulate neoplastic cell survival, proliferation, and migration by leukocytes is well recognized [22, 27]. MCs produce PDGF, stem-cell factor (SCF) and transforming growth factor-alpha (TGFα), while TAMs can produce FGF, hepatocyte growth factor (HGF), EGFR ligands (including EGF and HB-EGF), as well as PDGF and TGFα [22]. In particular, leukocyte-derived TNFα has been found to enhance invasive/migratory phenotypes of breast, skin and ovarian cancer cells [61–64]. In breast cancer cell lines, TNFα regulates epithelial invasion through activation of downstream signaling cascades including Jun N-terminal kinase (JNK) and nuclear factor κB (NFκB) transcription factor. Activated JNK and NFκB in turn induce gene expression of pro-invasive factors such as EMMPRIN (extracellular matrix metallo-protease inducer) and MIF (migration inhibitory factor), whose expression enhances MMP-2 and -9 secretion and activity [61, 63, 64].

Moreover, a recent study by Luo and colleagues found that expression of leukocyte-derived inflammatory mediators during prostate carcinogenesis inhibited expression of known metastasis suppressor genes [65]. Tumor infiltrating T cells and macrophages present in malignant prostatic stroma produce the TNFα-related cytokine RANKL (Receptor Activator for NFκB Ligand). RANKL, through interaction with its' receptor RANK, activates Inhibitor of NFκB Kinase α (IKKα), that in-turn leads to transcriptional repression of the metastatic tumor suppressor gene maspin [66, 67]. When expressed, maspin inhibits metastasis by repressing neoplastic cell migration and invasion in part by altering the profile of integrins expressed by neoplastic cells [68]. However, loss of maspin regulation by IKKα leads to stabilization of maspin expression and significantly inhibits lymphatic and pulmonary metastasis of prostatic tumor cells [65]. These results indicate that tumor-associated leukocytes can potentate metastatic spread by repressing metastatic inhibitors such as maspin. Though RANKL has not been shown to directly repress maspin in breast cancers, RANKL-mediated RANK activation induces migratory behavior in both breast and prostate carcinomas [69] and maspin over-expression from a mammary epithelial promoter inhibited metastasis of SV40 Tag-induced mammary carcinomas [70]. In addition, inverse correlations between maspin expression and metastatic potential in breast and prostate cancer have been revealed [70, 71]. In metastatic prostate cancer, bioavailability of RANKL is promoted by MMP-7 production in osteoclasts and leads to bone resorption at the tumor bone barrier [72, 73]. Taken together, these findings indicate that inflammatory cell production of cytokines such as RANKL may be a general promoter of metastatic behavior in prostate or mammary carcinomas.

In addition to increasing the invasive potential of neoplastic cells, concentration gradients of growth factors established by leukocytes present in neoplastic stroma can coordinate tumor cell movements towards, and intravasation into, tumor-associated vasculature. For example, macrophages are the primary source of EGF in breast cancer microenvironments [74, 75]. EGF promotes invasion/chemotaxis and intravasation of breast carcinoma cells through its interaction with EGFR as demonstrated using an *in vivo* needle aspiration assay through engagement of cofilin-dependent actin polymerization [76, 77]. In addition, paracrine interactions between TAMs and breast carcinoma cells form positive feed-forward loops involving macro-

phage-expressed EGF and CSF-1 expressed by neoplastic cells, that together result in breast carcinoma cells exhibiting "high-velocity" polarized movement (chemotaxis) along collagen fibers towards blood vessels directed by perivascular macrophages [22, 34, 78]. More recently studies have directly visualized EGF- and CSF-1-dependent intravasation of breast carcinoma cells into blood vasculature *in vivo* [27]. Taken together, these studies reveal crosstalk between tumor "educated" leukocytes and neoplastic cells that collaboratively regulate malignant conversion, invasion and spreading of neoplastic cells into angiogenic vasculature and thereby potentiating metastatic dissemination.

2.3 Immune prepping of pre-metastatic sites

Once a malignant cell is circulating in hematogenous vasculature, what regulates its homing to or survival in ectopic sites? Studies using experimental metastasis assays with B16F1 melanoma cells predicted that while 87% of injected malignant cells arrest in capillary beds of secondary organs within 90 min, only 83% survive up to 3 days in liver parenchyma, while a mere ~2% are capable of forming micrometastatic foci with <0.2% retaining ability to activate angiogenic vasculature and develop into bona fide secondary tumor [79]. Studies such as these articulate the fact that development of metastases is truly a rare event and begs the question of what then regulates survival and proliferation of malignant cells that arrive at secondary sites?

Some clues into the later aspects of events regulating metastasis have implicated MMP-9 made by TAMs and alveolar VEGFR1$^+$ endothelial cells in microenvironmental remodeling necessary for metastatic cell survival in lung [80]. Using a mouse model of experimental metastasis formation, Hiratsuka et al. [80] reported that following recruitment to sites of primary tumor growth, TAMs circulate to distal organs. Distal organs exhibiting low-level expression of VEGFR1 fail to induce MMP-9 in response to TAM presence and are therefore not suitable environments for subsequent metastatic cell growth. On the other hand, distal organs that are VEGFR1-positive and contain a population of endothelial cells capable of inducing expression of MMP-9 above that supplied by circulating TAMs are 'fertile' sites for productive metastatic growth. While induced expression of the VEGFR1 ligand VEGF-A does not appear to be involved, presence of an active VEGFR1 tyrosine kinase domain is necessary; thus, it seems reasonable that activated MMP-9 releases matrix-sequestered VEGF-A rendering it bioavailable for interaction with its receptors as has been reported by Bergers et al. [29], thus, stimulating efficient vascular remodeling and angiogenesis necessary for metastatic cell growth and survival. Studies by Matrisian and colleagues have demonstrated MMP-9 derived from inflammatory cells (possibly neutrophils) present in pre-metastatic lung facilitates survival/establishment of early metastatic cells, but not growth of metastatic foci [81]. While MMP-9 derived from Kuffer cells in liver parenchyma, and not bone marrow-derived cells, facilitate ability of metastatic colon cancer tumor foci to grow [72]. Taken together these findings indicate that mechanisms by which premetastatic niches enhance metastatic outgrowth are organ and cancer type specific.

More recently, molecules have been identified that "bookmark" pre-metastatic niches [82]. Studies by Hiratsuka and colleagues found that release of VEGF, transforming growth factor (TGF)β and TNFα from the primary tumor site promoted expression of S100A8 and S100A9 in lung endothelium and myeloid cells [82]. Expression of S100 proteins subsequently facilitates homing of tumor cells to lung parenchyma. Interestingly, TAMs and MCs have also been found to be potent producers TNFα, TGFβ and VEGF [22, 83]. In this regard, inflammatory infiltrates at primary tumor sites may in part regulate formation of the premetastatic niche by releasing pro-inflammatory factors into the circulation. Certainly, recruitment of VEGFR1$^+$ hematopoietic progenitor cells and perhaps other myeloid cells to premetastatic sites may be capable of triggering the angiogenic switch necessary for metastatic cell survival and eventual outgrowth.

Lyden and collages similarly demonstrated that hematopoietic progenitor cells are recruited to pre-metastatic sites prior to arrival of malignant cells [84, 85]. These bone-marrow derived VEGFR1$^+$ cells home to pre-metastatic niches and appear to promote recruitment of tumor cells from blood and favor their outgrowth at metastatic sites. Interestingly, bone-marrow derived VEGFR1$^+$ cells are predominantly identified at sites of future metastatic colonization as opposed to other organs [84, 85]. For example, subcutaneous implantation of Lewis lung carcinoma cells and B16 melanoma cells recruited VEGFR1$^+$ cells to lung and liver, respectively—the sites where these tumor cells preferentially metastasize. Taken together these results indicate that factors derived from primary tumors, possibly inflammatory in nature, change the pre-metastatic microenvironment making it more hospitable to potential metastatic colonizers.

3 Speculation and future directions

It is clear that metastasis is a multi-step process where each step requires specific interactions between genetically modified neoplastic cells and activated host cells, thus supporting the notion that targeting host response pathways with anti-cancer therapeutics may be efficacious. Activated stromal cells and the processes they regulate make attractive therapeutic targets as they do not harbor a high degree of genetic

instability associated with evolving neoplastic cells that favors drug resistance. In addition, host-targeted agents can be harnessed as chemopreventative targets. However, it is important to recognize that host cells and the normal physiology they regulate are critical components of tissue and organ homeostasis; thus interfering with these normal functions can result in untoward side-effects as has been observed with both protease and COX-2 inhibitors in the clinic [86, 87]. Nevertheless, once we determine what risk–benefit ratio are acceptable, there are likely to be multiple situations where such host-targeted therapies can be used to patient's advantage enabling treating cancer as a chronic disease with the goal of extending patient survival as opposed to "curing" the patient and ridding them of malignant cells. When used in combination with standard anti-tumor approaches (cytotoxic drugs for example), host-targeted therapies may offer the possibility of an overwhelming assault that tumors will not easily survive. Alternatively, when dormant malignant disease is suspected, perhaps strategies should be considered that foster maintenance of immune and tissue homeostasis. Our current focus should be in determining optimum combinations of anti-tumor and anti-host therapies that will translate to significant survival and quality-of-life improvements for cancer patients.

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APPENDIX H

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Polarized immune responses differentially regulate cancer development

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Summary: Tumor-associated immune responses assert varied responses toward developing neoplasms that can either act to eradicate malignant cells via engagement of potent cytotoxic programs or alternatively enhance tumor growth through release of multifunctional pro-tumor mediators. Seemingly paradoxical, these disparate activities reflect a continuum of polarization (or activation) states possible for distinct leukocyte subsets that demonstrate tissue, organ, and tumor selectivity. Herein, we review clinical and experimental studies investigating cellular and molecular mechanisms utilized by neoplastic tissues to alternatively polarize immune responses that favor either pro- or anti-tumor immunity.

Keywords: *cancer, humoral immunity, inflammation, innate immunity, leukocytes, adaptive immunity*

Introduction: cancer and inflammation

The cellular composition of cancers include 'initiated' or neoplastic cells harboring genetic mutations, typically referred to as 'tumor' or 'cancer' cells, as well as diverse populations of genetically stable cell types that are activated and/or recruited to the local microenvironment, e.g. innate and adaptive immune cells, cells composing the hematogenous and lymphatic vasculature, fibroblasts, and other specialized mesenchymal support cells. Reciprocal interactions between responding 'normal' cells, their mediators, and structural components of extracellular matrix (ECM) with genetically altered neoplastic cells regulate all aspects of tumorgenicity (1–4). Clinical and experimental studies in the last decade have enhanced our understanding of how activated and/or recruited genetically stable cells contribute to tumorigenesis. For a neoplastic tissue to expand, not only do initiated tumor cells need to acquire unlimited self-renewal capacity (5), but they also need to co-opt cellular programs embedded within the tissue that enhance blood flow, oxygenation, and waste removal, i.e. angiogenesis and lymphangiogenesis, as well as molecular programs that favor tumor cell survival, and enhanced metabolism of soluble and insoluble components of ECM that favor remodeling and expansion of the tissue (4). While some experimental studies

have revealed the intrinsic properties of tumor cells that regulate aspects of these programs, also emerging has been the realization that innate (myeloid) and adaptive (lymphoid) immune cells functionally contribute toward all aspects of cancer development (2, 6, 7).

Innate immune cells, including mast cells, macrophages, granulocytes, dendritic cells (DCs), and natural killer (NK) cells, represent the first line of defense against pathogens and foreign agents. In response to disruption of tissue homeostasis, tissue-resident innate immune cells locally secrete soluble factors, such as cytokines and chemokines, matrix-remodeling proteins, and other bioactive mediators, that recruit additional leukocytes from the circulation into the tissue, i.e. inflammation. In response to pathogen infection, some recruited immune cells (also referred to as inflammatory cells) directly eliminate pathogenic agents *in situ*. Alternatively, professional antigen-presenting cells (APCs) including DCs and macrophages take up foreign antigens (including tumor antigens) and migrate to lymphoid organs where they 'present' their antigens to adaptive immune cells. Upon recognition of presented antigens, B lymphocytes, $CD8^+$ cytotoxic T lymphocytes (CTL), and $CD4^+$ T helper (Th) lymphocytes, mount an 'adaptive' immune response targeted against the foreign agent. By these mechanisms, acute activation of innate immunity sets the stage for activation of antigenically committed adaptive immune responses. Once foreign agents have been eliminated, inflammation resolves, and tissue homeostasis is restored. Regarding cancer risk and/or cancer development, it is now clear that similar immunological responses required for activating acute inflammation can be co-opted, such that if persistent inflammation is maintained in a tissue, it can instead promote neoplastic programming of that tissue and enhance cancer development (2, 6, 7).

The fact that some leukocytes promote – rather than restrict – tumor growth may be viewed as an apparent paradox (8, 9). Historically, leukocytes found in developing tumors were thought to represent an attempt by the host to eradicate transformed cells. Undeniably, certain leukocytes, such as some T lymphocyte subsets and NK cells, play a vital function in constraining tumor development (10), and it has been postulated that many more tumors arise than those that eventually develop to fully malignant disease owing to such activity. However, it is now clear that tumor-infiltrating leukocytes also play causal roles in cancer development and progression (2, 6–8, 11, 12). Herein, we review clinical and experimental studies investigating cellular and molecular mechanisms utilized by neoplastic tissues to alternatively polarize immune responses to favor either pro- or anti-tumor immunity.

Establishing pro-tumor immunity

In 1863, Virchow first postulated that cancer originates at sites of chronic inflammation, in part based on his hypothesis that some classes of irritants causing inflammation also enhance cell proliferation. When tissues are injured or exposed to chemical irritants, damaged cells are removed by induction of cell death pathways, while cell proliferation is enhanced to facilitate tissue regeneration in an attempt to re-establish tissue homeostasis. Proliferation and acute inflammatory responses are resolved only after insulting agents are removed and/or tissue repair completed. However, when insulting agent persists over time, so too do sustained cycles of cell proliferation/death and localized inflammation, and together these processes generate a microenvironment rich in inflammatory cell bioactive products that can increase neoplastic risk and foster tumor progression. Thus, while sporadic or inherited genetic mutations in critical genes regulating cell cycle, cell death, differentiation, metabolism, and adhesion represent initiating events in tumorigenesis ('initiation'), chronic inflammatory responses can promote full malignant potential of neoplastic cells ('promotion').

Pre-malignant and malignant tissues are known to be associated with changes in leukocyte profiles as well as their functionality (13, 14), including suppressed CTL responses associated with tumor rejection, in combination with enhanced humoral immunity (HI), that can promote tumor progression (13, 15). Distinctive $CD4^+$ T-cell subsets, e.g. Th1, Th2, or Th17 cells, secrete unique repertoires of cytokines that mediate their responses. Th1 cells produce interleukin-2 (IL-2) and interferon-γ (IFN-γ) for example, and direct CTL responses, whereas Th2 cells produce IL-4, IL-13, and IL-10 and facilitate local HI responses, whereas Th17 polarized cells produce IL-23, IL-6, and tumor necrosis factor-α (TNF-α), factors that promote and/or sustain chronic inflammation. In peripheral blood of patients with bladder and colorectal cancer, proportions of Th1 cells, identified by intracellular production of IFN-γ or IL-2, is decreased, whereas proportions of Th2 cells producing IL-4, IL-6, and/or IL-10 is significantly elevated, as compared with otherwise healthy cohorts (16, 17). A recent study investigating characteristics of leukocytic infiltrations in human cervical carcinomas found that $CD3^+$ tumor-infiltrating T cells display enhanced Th2 cytokine profiles and specifically increased IL-4 and reduced IFN-γ (18). In line with these findings, alterations in immune cell status (suppressed CTL responses and enhanced HI) have also been reported in chronic

inflammatory diseases associated with increased cancer risk (reviewed in 13). Taken together, these compelling clinical findings indicate that enhanced pro-tumor immune responses underlie increased risk for neoplastic progression in tissues afflicted with chronic inflammatory disease pathologies and/or tissues that harbor initiated neoplastic cells.

Polarization of Th cell responses

The specific immune response to a tumor is directed by interactions with mature APCs and the nature of the pro-inflammatory milieu (19). In this context, regulatory $CD4^+$ T cells play an important role in orchestrating responses. Naive T cells of the same antigenic specificity can be 'polarized' into distinct functional effector cells that depend upon early environmental signals received when antigen is presented (20). These signals are produced by innate leukocytes and cells at the site of injury and are registered by receptors expressed on naive T-cell precursors (21). Following viral infection, for example, infected cells rapidly release type-I interferons (IFN-α and/or β) (22), that activate early viral defense programs, and are also important for polarizing the immune system toward an anti-viral Th1 response (23).

Effector $CD4^+$ T cells of the Th1 lineage evolved to eradicate intracellular pathogens, such as intracellular bacteria and viruses, through activation of CTL responses, as well as induction of immunoglobulin G2a (IgG2a) and IgG3 production (24). In the context of cancer development, it has been postulated that some IFNs mediate anti-tumor immunity, in part through regulating polarized Th1 responses (25). IFN-induced initiation of Th1 cell polarization occurs when T-cell receptor (TCR) activation is accompanied by IFN-induced signaling through the signal transducer and activator of transcription 1 (STAT1) intracellular signaling cascade, the earliest step of Th1 polarization (26). Signaling through STAT1 upregulates expression of the IL-12Rβ2 chain of the IL-12 receptor, thus yielding cells responsive to IL-12, a cytokine important for further differentiation of Th1 cells (27). This process also stimulates IFN-γ production and induces expression of IL-18Rα (28). Mature Th1 effector cells can produce IFN-γ through TCR-dependent pathways but also are capable of producing the cytokine independently of antigen stimulation, if activated by IL-12 and IL-18 (29).

Cells of the Th2 lineage are thought to have evolved to enhance elimination of parasitic infections and are characterized by production of IL-4, IL-5, and IL-13. These cytokines are potent activators of IgE production and recruitment of eosinophils and granulocytes (30). Specification of Th2 cells is initiated by TCR signaling together with IL-4 signaling through STAT6, resulting in induced epigenetic chromatin remodeling in the Th2 cytokine cluster (31) while simultaneously suppressing STAT4 and IL-12Rβ2 expression (32). Together, these events favor expression of Th2-related cytokines, while rendering them insensitive to repolarization toward Th1 lineage commitment.

Recent studies have revealed a greater diversity in the $CD4^+$ T-cell effector repertoire and have linked the cytokines IL-23 and IL-17 to a new arm of the Th cell family, referred to as Th17 cells (33). Development of Th17 cells from naive T cells is induced by transforming growth factor-β (TGF-β) together with IL-6 as an important cofactor (34). TCR stimulation can directly stimulate Th17 cytokine production, while IL-1, IL-18, and IL-23 can potentiate this effect (24). $CD4^+$ Th17 cells have been implicated in clearance of extracellular bacteria and autoimmune disorders, such as experimental autoimmune encephalitis and collagen-induced arthritis, and are associated with recruitment of granulocytes and expression of IgM, IgG, and IgA (24, 35). Th17 responses have also been associated with aspects of cancer development (36). In a mouse model of chemically induced skin carcinogenesis, IL-23 was identified as an important mediator of the inflammatory response associated with promotion (36).

A fourth class of $CD4^+$ cells, namely regulatory T (Treg) cells, suppress effector functions of CTLs and have important physiological roles in preventing autoimmune disease and exacerbated immunity against infections (9, 14). Two main subsets of Treg cells have been identified: natural Treg cells that form in the thymus and adaptive Treg cells that differentiate in peripheral tissues. Natural Treg cells are $CD4^+CD25^+FoxP3^+$ and form centrally in the thymus by high-affinity TCR with antigens expressed in thymic stroma (14, 37). Natural Treg cells suppress immune responses through cell surface molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA4), membrane-bound TGF-β, and pericellular generation of adenosine. Adaptive Treg cells are $CD4^+CD25^+FoxP3^{+/low}$, form in peripheral tissues in the presence of IL-10 or TGF-β, and suppress immune responses mainly through release of soluble factors, such as IL-10 and TGF-β (24). *In vivo* depletion of Treg cells using neutralizing antibodies directed against CD25 enhance anti-tumor T-cell responses and induce regression of experimental tumors, e.g. sarcomas and melanomas (38, 39). Together with clinical observations revealing that presence of Treg cells in patients with ovarian cancer correlated with reduced survival (40), these findings may indicate their critical role in regulating pro- versus anti-tumor immunity.

Diverse $CD4^+$ T-cell subsets orchestrate a wide range of immune responses depending upon cues received from their

microenvironment that can enhance or limit pro- and/or anti-tumor immunity (*Fig. 1*). Understanding the tissue and organ-specific nuances of these signals may reveal important targets for anti-cancer immune-based therapy whose modulation would enhance anti-tumor immune response and thus eradication or at least stabilization of pre-malignant disease.

Stimulating a polarized Th response

Cell autonomous and cell non-autonomous mechanisms regulate Th polarization. One required signal is presentation of specific peptides by class II major histocompatibility complex molecules to TCRs (41) that effectively determine which tumor antigens immune responses are directed toward (42). Following engagement of TCRs, Th cells require costimulatory signals, such as CD40-CD40L and CD28-CD80/86 receptor-ligand interactions, from APCs to become activated. A well-characterized costimulatory receptor is CD28, which binds CD80 and CD86 on APCs, thereby stimulating survival and proliferation of Th cells (43). Both antigen-specific signals mediated through the TCR and antigen-non-specific costimulatory signals are necessary for T-cell activation. However, an additional and earlier signal is also necessary for APC activation that orientates subsequent Th responses. This signal is induced by ligation of pathogen-recognition receptors (PRRs) that recognize factors associated with infection and tissue damage (20). PRRs have recently been implicated as important for both anti- and pro-tumor activities by the immune system and are discussed further below.

Innate immune responses by resident and recruited leukocytes have historically been viewed as the first line of defense providing rapid non-specific protection to the local tissue/organ and thereby allowing time for adaptive immune response pathways to become engaged. However, it is now clear that adaptive immunity also depends on the level and

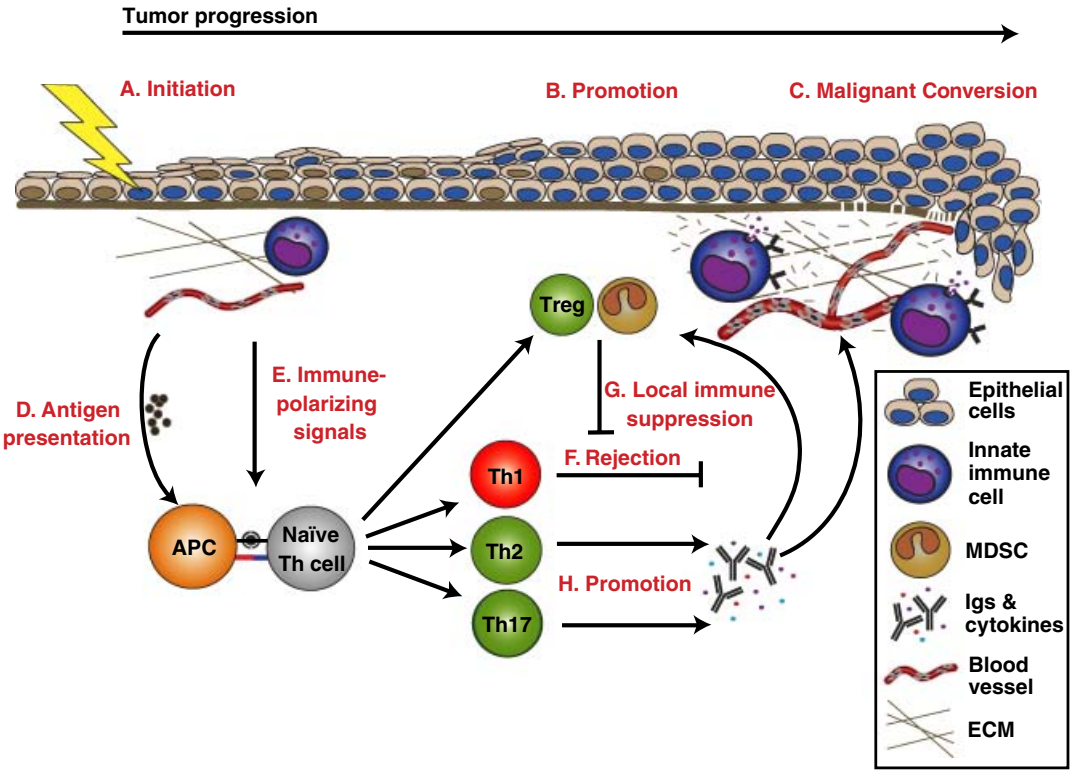

***Fig. 1.* Polarization of immune responses during tumor development**. Epithelial cancers develop through expansion of cells harboring mutations in key regulatory genes, i.e. initiated cells (A). Microenvironmental effectors, such as inflammation, promote (B) malignant conversion (C) of initiated cells. When an inflammatory response is triggered during initiation or promotion, antigen-presenting cells (APCs), such as dendritic cells and macrophages, take up tumor-associated antigens and present them (D) to naïve T helper (Th) cells. Depending on the context and the inflammatory microenvironment in which antigen presentation occurs, Th cells can become alternatively polarized. The process of immune polarization integrates a large number of microenvironmental signals (E) into one general outcome. Three discrete and mutually exclusive Th cell lineages, Th1, Th2, and Th17, have been defined. Th1 polarized immune responses (F) are associated with cytotoxic T lymphocytes (CTL)-mediated killing of tumor cells and favor cancer regression. CTL-mediated tumor cell cytotoxicity can be inhibited through recruitment and/or conversion of natural or adaptive regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (G). Th2 and Th17 polarized immune responses both have been shown to promote tumor progression (H) by establishing chronic inflammation, e.g. through cytokine production and induction of humoral responses. Chronic inflammation can promote tumor progression in multiple ways, such as stimulation of tumor cell proliferation and dedifferentiation, activation of angiogenesis, and recruitment of Tregs and MDSCs, as well as through remodeling of extracellular matrix (ECM) and tissue basement membranes.

specificity of the initial 'danger' signals perceived by activated innate leukocytes (21, 44). Following infection, innate leukocytes recognize substances produced by microbes through germline-encoded PRRs. The best-studied group of PRRs is the Toll-like receptors (TLRs). TLRs are type I integral membrane glycoproteins with cytoplasmic signaling domains homologous to that of the IL-1R (21). TLRs were initially investigated for their role in defense against microbes, but it is now clear that TLRs play an important role in polarizing immune responses during tumor progression. Twelve TLRs have been described, and each member has a unique pattern of cellular and tissue expression. TLRs are expressed on innate immune cells, including DCs, mast cells, macrophages, neutrophils, and granulocytes, but also on endothelial cells, epithelial cells, and fibroblasts (21). Following engagement, TLRs dimerize and undergo conformal changes required for recruitment of Toll-IL-1 resistance (TIR)-domain-containing adapter molecules to the TIR domain of the TLR. There are four adapter molecules, namely myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP)/MyD88-adapter-like (MAL), TIR-domain-containing molecule 1 (TICAM1), and TIR-domain-containing adapter inducing IFN-β (TRIF)-related adapter molecule (TRAM) (45).

It is now clear that distinct responses are elicited by different TLR ligands that can in part be explained by differential usage of adapter molecules. There are two main outcomes of these signaling pathways: the MyD88-dependent pathway that leads to production of pro-inflammatory cytokines and polarization toward a Th2 phenotype (46) or, alternatively, expression of type I IFNs and polarization toward a cytotoxic Th1 response (46).

Stimulation with TLR3, TLR4, TLR7, and TLR9 agonists, but not TLR2 agonists, leads to type I IFN production, and cytokines with potent anti-tumor effects including DC maturation, upregulation of the activation markers CD40, CD80, and CD86 and expression of IFN-α and IFN-β (33), giving rise primarily to anti-viral and Th1-polarized cytotoxic responses. This polarization can be either MyD88-dependent or independent for different TLRs. Stimulation of TLR9, for example by its endogenous ligand, free DNA, elicits strong Th1 responses that have demonstrated anti-tumor activity and are currently being tested in phase II and phase III clinical trials (47). Recently, Apetoh *et al.* (48) reported that following chemotherapy or radiation, dead tumor cells release endogenous TLR ligands, such as high-mobility group box 1 protein (HMGB1). HMGB1 activates TLR4, which activates DCs through a MyD88-dependent pathway and promotes an anti-tumor CTL response (48). The authors further demonstrated that breast cancer patients with a mutation in TLR4, rendering it unable to bind HMGB1, harbor increased risk for development of metastatic disease (48).

Other TLRs can elicit pro-inflammatory responses that are largely mediated through MyD88. MyD88 participates in signaling downstream of IL-1R and all TLRs except TLR3 (49). Stimulation of this pathway recruits MyD88 and TIRAP to the receptor and the subsequent activation of the nuclear factor κB (NF-κB) transcription factor, resulting in production of pro-inflammatory cytokines such as IL-6 and IL-12 (21). Two recent papers demonstrated that MyD88-dependent pathways are important for promotion of tumor progression in inflammation-associated carcinomas (50, 51). Karin and colleagues (51) found that MyD88-dependent upregulation of IL-6 following administration of a hepatic carcinogen and ablation of MyD88 prevented IL-6 upregulation and protected mice from tumor development. Rakoff-Nahoum and Medzhitov (50) investigated intestinal tumorigenesis and found that MyD88 was required for progression from micro- to macro-adenomas, while tumor initiation was unaffected. Signaling through TLR4 and MyD88 is also important for induction of HI responses toward human papilloma virus (HPV)-16 capsid proteins, a virus that is associated with cervical cancer in humans (52). Interestingly, TLRs not only respond to pathogen-derived substances but also to endogenous ligands often associated with tissue damage or tumor development (53). TLR4, for example, responds to extravasated fibrin, heparan sulfate, fibronectin, and hyaluronate (54); these ligands are commonly found in tumor stroma (55, 56). Taken together, tumor antigens are well known to trigger immune responses, which depending upon context and vigor, can either foster or inhibit pro-tumor immunity

Pro-tumor HI

B lymphocytes are the central component of HI and exert their effector function through antibody production, antigen presentation, and secretion of pro-inflammatory cytokines. In the context of cancer development, B cells have also been found to inhibit Th1-mediated anti-tumor immune responses (13). In experimental mouse models of lung adenocarcinoma, B-cell deficiency significantly enhanced therapeutic efficacy when treated with combinations of chemotherapy and IL-15, a Th-1 cytokine with IL-2-like anti-tumor bioactivities (57). In a syngeneic mouse xenograft model of colorectal cancer, partial B-cell depletion resulted in significantly reduced tumor burden (58). Moreover, in a mouse model of melanoma, the absence of B cells was associated with increased therapeutic efficacy of a

melanoma vaccine, with enhanced tumor protection in B-cell-deficient mice characterized by increased magnitude and longevity of specific cellular immune responses provoked by vaccination (59). In addition, B-cell-deficient mice exhibit resistance to several histologically diverse primary syngeneic tumors, associated with enhanced anti-tumor Th1 cytokines and CTL responses (60). In these studies, tumor growth was restored by adoptive transfer of B lymphocytes but not serum from wildtype mice to B-cell-deficient mice, accompanied by reduced Th1 cytokine levels and CTL response, indicating antibody-independent mechanisms mediating the response (60). Together, these experimental studies support the concept that B cells limit anti-tumor immunity through inhibition of Th1 and CTL responses while simultaneously bolstering Th2-effector cell pro-tumor functions.

Antibodies directed toward tumor-associated antigens are commonly found in cancer patients, e.g. c-myc, HER-2/neu, and p53 (61). However, production of these autoantibodies does not confer protection but paradoxically, correlates with poor prognosis and decreased survival for several human cancer types (62). Antibodies directed toward tumor antigens are thought to enhance tumor growth by promoting pro-tumor inflammatory responses and in general protecting tumor cells from CTL-mediated killing (63). In addition, extravasation of antigen-specific Ig into tumor stroma results in formation of immune complexes (ICs) that engender tumor-promoting inflammatory responses (8, 64). Ig-IC formation is indeed a common feature of cancer development. High levels of circulating ICs are associated with increased tumor burden and poor prognosis in patients with breast, genitourinary, and head and neck malignancies (65–67), and Ig deposition in neoplastic stroma has been reported in pre-malignant and malignant human breast and prostate tissues (8).

ICs have long been suspected as acting as initiators of inflammatory cascades associated with tissue destruction in autoimmune disease; however, underlying molecular mechanisms have remained elusive (68). These mechanisms have recently been investigated using an experimental mouse model of airway remodeling following *Mycoplasma pulmonis* infection. This study demonstrated that peripheral B-cell responses and local Ig-IC deposition are critical triggers for recruiting innate leucocytes into infected airways, that subsequently activate pro-angiogenic and tissue remodeling pathways necessary for resolving infection (69). As potentiators of inflammation associated with cancer development, B-cell activation is significant. Using a spontaneous transgenic mouse model of squamous carcinogenesis, i.e. K14-HPV16 mice (70), we reported that combined B- and T-lymphocyte deficiency eliminated IC deposition and attenuated innate immune cell infiltration into pre-malignant skin (71). As a consequence of deficient adaptive immunity and abated innate immune cell responses, there was an overall decrease in tissue remodeling activity, failure to activate angiogenic vasculature, retention of terminal differentiation programs in skin keratinocytes, and a 43% reduction in squamous cell carcinoma incidence (71). Adoptive transfer of B-lymphocytes or serum isolated from HPV16 mice (but not from wildtype naive mice) into B- and T-lymphocyte-deficient/HPV16 mice restored IC deposition, chronic innate immune cell infiltration in pre-malignant skin, and reinstated parameters for full malignancy, i.e. inflammation, tissue remodeling, angiogenesis, and keratinocyte hyperproliferation (71). These data indicate that B-lymphocyte-derived factors in serum, possibly Ig, are essential for establishing chronic inflammatory pathways that promote tumor development. In support of this concept, antibodies directed toward tumor antigens are known to enhance outgrowth and invasion of murine and human tumor-cell xenografts by recruiting and activating granulocytes and macrophages (64), important sources of vascular endothelial growth factor (72) that stimulates angiogenesis. Thus, serum proteins (presumably antibodies) produced by B cells are, at least in some scenarios, crucially involved in initiation of chronic inflammation that in addition to resolving acute tissue damage also are essential for promoting tumor development in neoplastic tissues.

Mechanisms by which ICs promote inflammation are still not fully understood; however, IgG Fc receptors (FcγRs), especially FcγRIII, and complement factors, in particular C5a, are recognized as co-dominant effectors in this process (73). Different types of FcγRs either activate or inhibit immune responses. In humans, three types of FcγRs exist: FcγRI/CD64, FcγRII/CD32, and FcγRIII/CD16. FcγRII is further subdivided into FcγRIIA, FcγRIIB, and FcγRIIC. Mice express a fourth FcγR, FcγRIV (13). Both mice and humans express a single inhibitory FcγR, FcγRIIB. Upon ligation of FcγRs with IgG that are complexed with antigen, different cellular responses are triggered depending on which receptors are engaged (13). FcγRI and FcγRIII, predominantly FcγRIII, mediate immune cell activation via their FcRγ chain that contains an immunoreceptor tyrosine-based activating motif (ITAM). ITAM-mediated activation triggers oxidative bursts, cytokine release, and phagocytosis by macrophages, antibody-dependent cell-mediated cytotoxicity by NK cells, and mast cell degranulation (68). Conversely, engagement of FcγRIIB, which instead contains immunoreceptor tyrosine-based inhibitory motifs, inhibits these same inflammatory responses

(68). A further complication is that immune cells frequently co-express both activating and inhibitory receptors, and the net cellular response is determined by a balance between activating and inhibitory signals. Regulatory functions of FcγRs have been studied using genetically engineered mouse models. Mice deficient in the FcRγ chain or activating type FcγRs are resistant to a wide range of hypersensitive reactions, such as vasculitis, glomerulonephritis, and skin Arthus reaction (68). In contrast, mice deficient in FcγRIIB exhibit enhanced IC-mediated inflammatory responses (68).

Nimmerjahn and Ravetch (74, 75) utilized *in vivo* activities of various IgG isotypes, in combination with determining their affinity for different FcγR, as a predictor of cellular response. In this scheme, affinities of IgGs to FcγRs are determined in the context of an activation-to-inhibitory ratio (A/I ratio), calculated by dividing affinity of an IgG subclass for the relevant activating receptor (FcγRIII or FcγRIV, depending on IgG isotype) by the affinity for the inhibitory FcγRIIB (74). IgG2a subclasses demonstrate the highest A/I ratio, followed by IgG2b and IgG1 having the lowest, whereas IgG3 demonstrates no detectable binding. The functional significance of these ratios were assessed *in vivo*, where it was found that IgG2a, the IgG with the highest A/I ratio, enhanced clearing of lung metastases in mice when injected with tumor cells; thus, the response correlated with the ratio. In additional experiments, A/I ratios were modified by altering IgG glycosylation profiles, and once again, antibodies with higher A/I ratios exhibited enhanced *in vivo* efficacy – effects that were attenuated in mice deficient in FcγRs but unchanged in mice deficient in components of the complement system, demonstrating that *in vivo* activity of IgGs, at least in the experimental setting, depends on FcγRs and not on complement activation (74). When investigated further, it was found that glycolytic modifications through sialylation of Fc regions of IgG reduce binding affinities toward FcγRs and thereby inhibit pro-inflammatory activities of IgG, and in contrast, reduction of sialylation, upon antigen challenge, switches the immune response pathway from anti-inflammatory to pro-inflammatory via differential engagement with FcγRs on effector cells (76). Together, these studies demonstrate functional importance of FcγRs in inflammation and autoimmune diseases; their role(s) during tumor development remains to be determined.

Inhibiting anti-tumor immunity

The predominant role of the mammalian immune system is to protect the body against infectious agents and to facilitate healing after injury; thus, the dogma has been that immune cells would similarly offer protection against primary tumor development and/or metastases. In support of this are clinical data revealing that patients taking immunosuppressive drugs or suffering from various types of immune deficiency disorders exhibit increased risk for some viral- and/or carcinogen-associated cancers (8, 77), thus indicating that absence of anti-viral immunity, presumably CTLs, affects relative cancer risk. However, for tumors not commonly associated with carcinogen exposure or viral infection (e.g. prostate, breast, ovarian, and uterine cancer), relative risk for cancer development is decreased in similar cohorts of immunocompromised individuals (8). Thus, an apparent paradox exists, where both anti- and pro-tumor immune programs can be engaged depending on organ specificity and cancer etiology.

Lymphocytes and some innate immune cells possess potent anti-cancer activities that can affect growth and/or metastatic spread. Recently, $CD3^+$ T-cell densities within colorectal cancer biopsies were found to be a better predictor of patient survival than standard histopathological staging methods (78). Similarly, infiltration of NK cells in human gastric or colorectal carcinoma is associated with a favorable prognosis (79). NK cells can play a role in protection against experimental tumor growth by directly killing tumor cells and indirectly by producing mediators with anti-angiogenic properties (80, 81).

The idea that neoplasms can be recognized and attacked by the adaptive immune system has encouraged several groups to attempt to activate adaptive immune cells to achieve anti-tumor immune responses (82). $CD8^+$ T cells were found to be particularly important and required for anti-tumor effects in several experimental mouse models (77). Furthermore, CTLs were able to eliminate only tumor cells expressing their cognate antigen, indicating a specific immune response (82). A subset of innate immune cells may contribute to CTL suppression, namely myeloid-derived suppressor cells (MDSCs), which are $CD11b^+Gr1^+$ cells and accumulate in peripheral blood of cancer patients (83, 84) as well as in tumors and lymphoid organs (9, 84–86). MDSCs are known to induce T-lymphocyte dysfunction by direct cell–cell contact and by production of immunosuppressive mediators, e.g. arginase and TGF-β production (84, 86, 87). TGF-β also converts naive $CD4^+$ T cells into adaptive Treg cells (14), indicating that MDSCs can inhibit anti-tumor adaptive immunity directly, as well as through polarization of local immune responses. In addition, MDSCs can promote tumor growth by contributing to tumor-associated angiogenesis (88). Together, these studies support the concept that B and

T lymphocytes can contribute to inhibition of Th1 and CTL responses and thereby aid tumor development.

Conclusion

B cells are crucial in defense against extracellular bacteria and parasites. They are also known to initiate autoimmune diseases through several mechanistic pathways, including production of autoantibodies, formation of ICs, activation of DCs and T cells, as well as cytokine production (89). Cancer patients contain elevated levels of serum Igs, some with anti-tumor specificity, but the role of these antibodies have been debated. Initially, they were assumed to represent an anti-tumor response; however, their presence often instead correlates with poor prognosis. This observation in combination with experimental studies demonstrating that treatment with tumor-specific B cells promote tumor progression, while also suppressing anti-tumor CTL responses has fostered a shift in thinking regarding the role of B cells and HI during cancer development (13, 60, 71, 90). Are there clinical implications for these more recent points of view?

In autoimmune disease, therapeutic depletion of B cells using a chimeric monoclonal antibody specific for human CD20, i.e. rituximab, in patients with rheumatoid arthritis, systemic lupus erythematosus, and others, have demonstrated clinical efficacy (91, 92). Rituximab has also proven to be clinically effective in adult acute lymphoblastic leukemia in combination with chemotherapy (93). Could rituximab then potentially be used therapeutically for treatment of solid tumors? A limited clinical study has been performed where advanced colon cancer patients were treated with rituximab (58). In these individuals, numbers of CD21-hyperpositive lymphocytes were reduced in parallel with a 50% reduction in tumor burden with no side effects due to therapy (58). Even though rituximab effectively deletes the vast majority of circulating B cells, no increased susceptibility to infection has been reported in treated patients (91), likely due to the fact that circulating concentrations of Igs are unchanged by rituximab, because memory B cells and/or plasma cells are not targets for rituximab. The efficacy of rituximab in safely manipulating HI as a therapeutic strategy is encouraging but is as of yet untested.

Clinical data as well as experimental animal studies support the notion that a sustained humoral response has the ability to elicit significant pro-tumor effects in developing neoplasms. Established tumors represent formidable opponents that often develop resistance toward available drug options. To develop new treatment strategies, one promising approach is to combine drugs that act on neoplastic cells together with compounds that disassemble microenvironmental support programs utilized by tumors for survival and spread. In this context, a more thorough understanding of immune-based modulation of tumor development will be instrumental for development of new treatment strategies for cancer.

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APPENDIX I

AACR Mouse Models of Cancer, Cambridge, MA, USA

Microenvironmental Regulation of Vascular Homeostasis and Leakage

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Assembly and maintenance of mature tubular networks for blood circulation is crucial to all mammals. The three major components of these networks are endothelial cells, vascular smooth muscle cells and extracellular matrix (ECM). Under homeostatic conditions, these networks are maintained in quiescent states that foster vessel stability. Following physiologic or pathologic stimulation however, innate programs are rapidly activated that enable vascular remodeling. Despite many advances in the fields of vasculogenesis and angiogenesis, relatively little is known about molecular and cellular mechanisms that maintain vessel stability in homeostatic tissues and/or regulate extravasation of plasma proteins into interstitial tissue compartments following acute or chronic tissue damage. Moreover, little is known regarding how these pathways are altered is diseases where ECM molecules contribute to tissue function and/or underlie disease pathogenesis. Thus, identification of molecules/factors/pathways that regulate vascular stability under homeostatic, physiologic and pathologic tissue remodeling states, would yield important insights into potential targets for therapy in diseases where vessel stability and leakage are otherwise compromised. This presentation will discuss insights into the significance of ECM as a regulator of vessel stability and our identification of a novel pericellular pathway involving type I collagen, matrix metalloproteinase-14 and TGFβ that together regulate vascular homeostasis and response to tissue damage.

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International Society for Oncodevelopmental Biology and Medicine (ISOBM) Meeting, Pasadena, CA, USA

Inflammation, Proteases and Cancer Development

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Abstract

During the early development of cancer, many physiological processes occur in the vicinity of 'young tumor cells' that are similar to processes that occur during embryonic development and to healing of wounds in adult tissue, e.g., inflammation, angiogenesis and tissue remodeling (Coussens and Werb, 2002). During wound healing, inflammatory cells are recruited to sites of injury to eliminate potential bacterial infection as well as to facilitate healing by providing growth factors and proteases that are essential to the process. In so doing, a new blood supply is also formed that further helps the tissue heal. When 'healing' is complete, inflammation resolves and the tissue returns to its former state. Several of these parameters are conserved during tumor development; however, instead of initiating a 'healing' response, immune cells that infiltrate premalignant tissue provide growth-promoting factors and proteolytic enzymes that promote tumor development (Balkwill et al., 2005; Coussens and Werb, 2002). These observations are significant in light of the fact that individuals suffering from chronic inflammatory diseases harbor a greatly increased risk for cancer development in tissues infiltrated by activated leukocytes (de Visser et al., 2006), and indicate that by identifying molecular mediators regulating onset, activation and maintenance of inflammation in the neoplastic microenvioronement, we will reveal regulatory events/molecules that can be effectively targeted with anti-cancer therapeutics.

In trying to understand how chronic inflammation in premalignant tissues potentiates cancer development, we have genetically manipulated the host immune response and/or presence of various leukocyte-derived proteases (Coussens et al., 2000) in a transgenic mouse model of epithelial carcinogenesis, e.g. K14-HPV16 mice (Coussens et al., 1996). In so doing, we have found that inflammatory mast cells and granulocytes contribute in a dominant manner to the activation of neoplasia-associated tissue remodeling, angiogenesis and epithelial hyperproliferation and overall cancer incidence via their release of proteolytic enzymes of the cysteine, serine and metallo classes. Moreover, we have identified a critical regulatory pathway underlying persistent recruitment of innate immune cells toward developing neoplasms. We found that genetic elimination of mature T and B lymphocytes limited neoplastic progression to development of benign epithelial hyperplasias that failed to recruit innate immune cells into premalignant tissue (de Visser et al., 2005). Adoptive transfer of B lymphocytes or serum from K14-HPV16 mice into T and B cell-deficient/HPV16 mice was sufficient to restore innate immune cell infiltration into premalignant tissue and to reinstate necessary parameters for full malignancy, e.g., chronic inflammation, angiogenic vasculature and hyperproliferaive epidermis. These findings support a model in which B lymphocytes and/or activation of humoral immune responses in peripheral tissues is required for establishing chronic inflammatory states that promote de novo carcinogenesis, and support the concept that oncogene expression in 'initiated' cells alone is not sufficient for full malignant progression (Bissell and Radisky, 2001). Instead, additional signals provided by adaptive and innate immune cells are required for elaboration of the malignant state. Our results, in combination with results from other investigators, suggest that pharmacological interventions targeting activation and/or recruitment of innate immune cells towards premalignant tissue represent viable cancer

chemopreventative strategies. To be presented, will be recent results evaluating molecular mechanisms underlying humoral-activation of innate immune cells in K14-HPV16 transgenic mice and identification of critical proteolytic pathways regulating early activation of angiogenic responses and sustained immune cell infiltration of neoplastic tissue.

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18th Annual Pezcoller Symposium, Trento, ITALY

Immune Cells as Targets for Cancer Prevention

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Abstract

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In trying to understand how chronic inflammation in premalignant tissues potentiates cancer development, we have genetically manipulated the host immune response and/or presence of various leukocyte-derived proteases (Coussens et al., 2000) in a transgenic mouse model of epithelial carcinogenesis, e.g. K14-HPV16 mice (Coussens et al., 1996). In so doing, we have found that inflammatory mast cells and granulocytes contribute in a dominant manner to the activation of neoplasia-associated tissue remodeling, angiogenesis and epithelial hyperproliferation and overall cancer incidence via their release of proteolytic enzymes of the cysteine, serine and metallo classes. Moreover, we have identified a critical regulatory pathway underlying persistent recruitment of innate immune cells toward developing neoplasms. We found that genetic elimination of mature T and B lymphocytes limited neoplastic progression to development of benign epithelial hyperplasias that failed to recruit innate immune cells into premalignant tissue (de Visser et al., 2005). Adoptive transfer of B lymphocytes or serum from K14-HPV16 mice into T and B cell-deficient/HPV16 mice was sufficient to restore innate immune cell infiltration into premalignant tissue and to reinstate necessary parameters for full malignancy, e.g., chronic inflammation, angiogenic vasculature and hyperproliferaive epidermis. These findings support a model in which B lymphocytes and/or activation of humoral immune responses in peripheral tissues is required for establishing chronic inflammatory states that promote de novo carcinogenesis, and support the concept that oncogene expression in 'initiated' cells alone is not sufficient for full malignant progression (Bissell and Radisky, 2001). Instead, additional signals provided by adaptive and innate immune cells are required for elaboration of the malignant state. Our results, in combination with results from other investigators, suggest that pharmacological interventions targeting activation and/or recruitment of innate immune cells towards premalignant tissue represent viable cancer chemopreventative strategies. To be presented, will be recent results evaluating immuogobulin-activation

of innate immune cells in K14-HPV16 transgenic mice and identification of the lysosomal cysteine protease, e.g., cathepsin C, as a critical mediator of inflammation and angiogenesis associated with epithelial cancer development.

LMC is supported by grants from the NIH (CA72006, CA94168, CA098075), the Sandler Program in Basic Sciences, National Technology Center for Networks and Pathways (U54 RR020843), and a Department of Defense Era of Hope Scholar Award (BC051640)

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INFLAMMATION, PROTEOLYSIS AND CANCER DEVELOPMENT

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During the early development of cancer, many physiological processes occur in the vicinity of 'young tumor cells' that are similar to processes that occur during embryonic development and to healing of wounds in adult tissue, e.g., inflammation, angiogenesis and tissue remodeling[1]. During wound healing, inflammatory cells are recruited to sites of injury to eliminate potential bacterial infection as well as to facilitate healing by providing growth factors and proteases that are essential to the process. In so doing, a new blood supply is also formed that further helps the tissue heal. When 'healing' is complete, inflammation resolves and the tissue returns to its former state. Several of these parameters are conserved during tumor development; however, instead of initiating a 'healing' response, immune cells that infiltrate premalignant tissue provide growth-promoting factors and proteolytic enzymes that promote tumor development[1, 2]. These observations are significant in light of the fact that individuals suffering from chronic inflammatory diseases harbor a greatly increased risk for cancer development in tissues infiltrated by activated leukocytes[3], and indicate that by identifying molecular mediators regulating onset, activation and maintenance of inflammation in the neoplastic microenvioronement, we will reveal regulatory events/molecules that can be effectively targeted with anti-cancer therapeutics.

In trying to understand how chronic inflammation in premalignant tissues potentiates cancer development, we have genetically manipulated the host immune response and/or presence of various leukocyte-derived proteases[4] in a transgenic mouse model of epithelial carcinogenesis, e.g. K14-HPV16 mice[5]. In so doing, we have found that inflammatory mast cells and granulocytes contribute in a dominant manner to the activation of neoplasia-associated tissue remodeling, angiogenesis and epithelial hyperproliferation and overall cancer incidence via their release of proteolytic enzymes of the cysteine, serine and metallo classes. Moreover, we have identified a critical regulatory pathway underlying persistent recruitment of innate immune cells toward developing neoplasms. We found that genetic elimination of mature T and B lymphocytes limited neoplastic progression to development of benign epithelial hyperplasias that failed to recruit innate immune cells into premalignant tissue[6]. Adoptive transfer of B lymphocytes or serum from K14-HPV16 mice into T and B cell-deficient/HPV16 mice was sufficient to restore innate immune cell infiltration into premalignant tissue and to reinstate necessary parameters for full malignancy, e.g., chronic inflammation, angiogenic vasculature and hyperproliferaive

epidermis. These findings support a model in which B lymphocytes and/or activation of humoral immune responses in peripheral tissues is required for establishing chronic inflammatory states that promote de novo carcinogenesis, and support the concept that oncogene expression in 'initiated' cells alone is not sufficient for full malignant progression[7]. Instead, additional signals provided by adaptive and innate immune cells are required for elaboration of the malignant state. Our results, in combination with results from other investigators, suggest that pharmacological interventions targeting activation and/or recruitment of innate immune cells towards premalignant tissue represent viable cancer chemopreventative strategies. To be presented, will be recent studies evaluating molecular mechanisms underlying humoral-activation of innate immune cells during premalignancy in K14-HPV16 transgenic mice and identification of critical proteolytic pathways regulating early activation of angiogenic responses and sustained immune cell infiltration of neoplastic tissue.

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Specialty and Present Interest:
Mouse models of cancer, Tumor microenvironment, tumor-associated inflammation, angiogenesis, extracellular proteases

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2007 AAAS Annual Meeting, San Francisco, CA USA

INFLAMMATION, PROTEOLYSIS AND CANCER DEVELOPMENT

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In trying to understand how chronic inflammation in premalignant tissues potentiates cancer development, we have genetically manipulated the host immune response and/or presence of various leukocyte-derived proteases[1] in a transgenic mouse model of epithelial carcinogenesis, e.g. K14-HPV16 mice[2]. In so doing, we have found that inflammatory mast cells and granulocytes contribute in a dominant manner to the activation of neoplasia-associated tissue remodeling, angiogenesis and epithelial hyperproliferation and overall cancer incidence via their release of proteolytic enzymes of the cysteine, serine and metallo classes. Moreover, we have identified a critical regulatory pathway underlying persistent recruitment of innate immune cells toward developing neoplasms. We found that genetic elimination of mature T and B lymphocytes limited neoplastic progression to development of benign epithelial hyperplasias that failed to recruit innate immune cells into premalignant tissue[3]. Adoptive transfer of B lymphocytes or serum from K14-HPV16 mice into T and B cell-deficient/HPV16 mice was sufficient to restore innate immune cell infiltration into premalignant tissue and to reinstate necessary parameters for full malignancy, e.g., chronic inflammation, angiogenic vasculature and hyperproliferaive epidermis. These findings support a model in which B lymphocytes and/or activation of humoral immune responses in peripheral tissues is required for establishing chronic inflammatory states that promote de novo carcinogenesis, and support the concept that oncogene expression in 'initiated' cells alone is not sufficient for full malignant progression[4]. Instead, additional signals provided by adaptive and innate immune cells are required for elaboration of the malignant state. Our results, in combination with results from other investigators, suggest that pharmacological interventions targeting activation and/or recruitment of innate immune cells towards premalignant tissue represent viable cancer chemopreventative strategies. To be presented, will be recent studies evaluating molecular mechanisms underlying humoralactivation of innate immune cells during premalignancy in K14-HPV16 transgenic mice and identification of critical pathways regulating early activation of angiogenic responses and sustained immune cell infiltration of neoplastic tissue.

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7th AACR-Japanese Cancer Association Joint Conference, Waikaloa, Hawaii, USA

INFLAMMATION, PROTEOLYSIS AND CANCER DEVELOPMENT

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The Second International Symposium on Cancer Metastasis and the Lymphovascular System: Rational Basis for Therapy, San Francisco CA USA

INFLAMMATION, HUMORAL IMMUNITY AND EPITHELIAL CANCER DEVELOPMENT

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2007 Keystone Symposium on Mechanisms Linking Inflammation and Cancer, Santa Fe, New Mexico. USA

Inflammation and Cancer

Organizers: Lisa M. Coussens, Glenn Dranoff and Fran Balkwill

Our appreciation of the complexity of tumor biology has led us from considering tumors as autonomous masses of mutant cells to an awareness of tumors as entities that can hijack and exploit various normal physiologic processes of the host. The frequent presence of inflammatory cell infiltrates and chemical mediators of inflammation in tumors has been recognized for over a century, although an understanding of their role during cancer development has been elusive. Leukocytic infiltrates and cytokine/chemokine networks in premalignant tissues and tumors can be distinct depending upon the stage of malignant development and organ microenvironment. Current thinking is that activated immune cells provide both anti- and pro-tumorigenic signals, thus representing **targets** to be harnessed or attacked for therapeutic advantage depending upon environmental and/or cellular context. T lymphocytes are being exploited for their ability to induce tumor regression, as are strategies that disable innate immune cells or neutralize immunosuppressive or pro-inflammatory microenvironments. Such approaches may also provide clinical benefit for at-risk cancer patients and those with pre-malignant lesions. This meeting will link innate and adaptive immune regulatory mechanisms with cancer development and cancer treatment by focusing on relevant basic research, preclinical, translational and clinical studies.

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International Cancer Microenvironment Society (ICMS) - American Association of Cancer Research, 4th International Conference on Tumor microenvironment. Florence ITALY

INFLAMMATION, HUMORAL IMMUNITY AND CANCER DEVELOPMENT

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In trying to understand how chronic inflammation in premalignant tissues potentiates cancer development, we have genetically manipulated the host immune response and/or presence of various leukocyte-derived proteases[1] in a transgenic mouse model of epithelial carcinogenesis, e.g. K14-HPV16 mice[2]. In so doing, we have found that inflammatory mast cells and granulocytes contribute in a dominant manner to the activation of neoplasia-associated tissue remodeling, angiogenesis and epithelial hyperproliferation and overall cancer incidence via their release of proteolytic enzymes of the cysteine, serine and metallo classes. Moreover, we have identified a critical regulatory pathway underlying persistent recruitment of innate immune cells toward developing neoplasms. We found that genetic elimination of mature T and B lymphocytes limited neoplastic progression to development of benign epithelial hyperplasias that failed to recruit innate immune cells into premalignant tissue[3]. Adoptive transfer of B lymphocytes or serum from K14-HPV16 mice into T and B cell-deficient/HPV16 mice was sufficient to restore innate immune cell infiltration into premalignant tissue and to reinstate necessary parameters for full malignancy, e.g., chronic inflammation, angiogenic vasculature and hyperproliferaive epidermis. These findings support a model in which B lymphocytes and/or activation of humoral immune responses in peripheral tissues is required for establishing chronic inflammatory states that promote de novo carcinogenesis, and support the concept that oncogene expression in 'initiated' cells alone is not sufficient for full malignant progression[4]. Instead, additional signals provided by adaptive and innate immune cells are required for elaboration of the malignant state. Our results, in combination with results from other investigators, suggest that pharmacological interventions targeting activation and/or recruitment of innate immune cells towards premalignant tissue represent viable cancer chemopreventative strategies. To be presented, will be recent studies evaluating molecular mechanisms underlying humoral-activation of innate immune cells during premalignancy in K14-HPV16 transgenic mice and identification of critical pathways regulating early activation of angiogenic responses and sustained immune cell infiltration in neoplastic tissue.

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2006 AACR Workshop in Cancer Research: Pathology of Cancer. Snowmass. CO

The role of inflammatory cells in controlling the normal breast epithelial ducal polarity.

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Breast Cancer is the second leading cause of cancer related mortality among women in the United States and will lead to the deaths of twenty thousand women this year alone. These statistics highlight the need for development of more efficacious drugs aimed at treatment of malignant disease as well as the prevention of pre-malignant disease. For many years, cancer research has focused on the identification of tractable drug targets on or in malignant cells themselves. However, these cells are very heterogeneous and readily develop drug resistance. During the development of cancer, the microenvironments in which pre-malignant or at-risk cells develop effects their progression to the malignant state. Thus, our hypothesis is that the stromal microenvironment contains efficacious drug targets that can be harnessed for chemoprevention and/or intervention of breast cancer. In recent years, there has been increasing clinical and experimental evidence supporting a link between inflammation and breast cancer progression. While it was initially believed that leukocytic infiltrations in and around developing neoplasms represented an attempt of the host to eradicate neoplastic cells, it is now clear that individuals prone to chronic inflammatory diseases have an increased risk of developing breast cancer. We have examined the presence of leukocytes during human breast cancer progression by immunohistochemical detection of CD45+ cells and revealed that as breast epithelial cells undergo progression to malignancy, the number of CD45+ leukocytes present in stroma increases in parallel with the development of breast cancer. To study the functional significance of these diverse leukocytes, we have similarly examined the profile of CD45+ leukocytes during mammary carcinogenesis in several mouse models, e.g., MMTC-PyMT, MMTV-neu, MMTV-Wnt1 and C3-Tag,. and are examining how chronically activated innate immune cells participate in cancer development. We have hypothesized that infiltrating immune cells stimulate tumor progression in part by disrupting the polarity of mammary ductal epithelium which otherwise suppresses aberrant cell proliferation. Our studies have focused on the role of tumor-educated macrophages and mast cells in disrupting the normal polarized epithelium in mammary gland ducts. We are utilizing organotypic 3-dimensional culture models to assess the effects of co-culture of specific inflammatory cells on mammary epithelial acini (ductal structure) polarity, proliferation, and apoptosis. Some of these effects may be dependent on the activation of extracellular proteases by macrophages and mast cells. We believe that studying the molecular mechanisms by which infiltrating immune cells stimulate the progression of premalignant lesions to undergo malignant transformation will identify pathways that can be targeted for the treatment and prevention of breast cancer.

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2007 Keystone Symposium on Mechanisms Linking Inflammation and Cancer, Santa Fe, New Mexico. USA

Macrophages Regulate Mammary Epithelial Ducal Polarity

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Breast Cancer is the second leading cause of cancer related mortality among women in the United States and will lead to the deaths of twenty thousand women this year alone. These statistics highlight the need for development of more efficacious drugs aimed at treatment of malignant disease as well as the prevention of pre-malignant disease. For many years, cancer research has focused on the identification of tractable drug targets on or in malignant cells themselves. However, these cells are very heterogeneous and readily develop drug resistance. During the development of cancer, the microenvironments in which pre-malignant or at-risk cells develop effects their progression to the malignant state. Thus, our hypothesis is that the stromal microenvironment contains efficacious drug targets that can be harnessed for chemoprevention and/or intervention of breast cancer. In recent years, there has been increasing clinical and experimental evidence supporting a link between inflammation and breast cancer progression. We have examined the presence of leukocytes during human breast cancer progression by immunohistochemical detection of CD45+ cells and revealed that as breast epithelial cells undergo progression to malignancy, the number of CD45+ leukocytes present in stroma increases in parallel with the development of breast cancer. To study the functional significance of these diverse leukocytes, we have similarly examined the profile of CD45+ leukocytes during mammary carcinogenesis in two mouse models of mammary carcinogenesis, e.g., MMTV-PyMT, MMTV-neu, and are examining how chronically activated innate immune cells participate in cancer development. We have hypothesized that infiltrating immune cells stimulate tumor progression in part by disrupting the polarity of mammary ductal epithelium which otherwise suppresses aberrant cell proliferation. Our studies have focused on the role of tumor-educated macrophages and mast cells in disrupting the normal and premalignant polarized mammary epithelium. We are utilizing organotypic 3-dimensional culture models to assess the effects of co-culture of inflammatory cells on mammary epithelial acini (ductal structure) polarity, proliferation, and apoptosis. We believe that studying the molecular mechanisms by which infiltrating immune cells stimulate progression of premalignant lesions to undergo malignant transformation will identify pathways that can be targeted for the treatment and prevention of breast cancer.

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2007 AACR, *Frontiers in Cancer Prevention Research Conference*, Philadelphia, PA, USA

Inflammation and Cancer: Organ-specific Regulation of Cancer Development

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The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have recently appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas chronic inflammation of premalignant skin neoplasms is B cell–dependent, during mammary carcinogenesis, T cells appear to play more of a dominant role in regulating pro-tumor and pro-metastatic properties of myeloid cells. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and thoughts on how these properties can be harnessed for effective anticancer therapeutics.

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2007 , 7th *International Symposium on Hodgkin Lymphoma*, Cologne, GERMANY

Title, authors and affiliation

Inflammation and Cancer: Organ-specific Regulation of Cancer Development

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During the early development of cancer, many physiological processes occur in the vicinity of 'young tumor cells' that are similar to processes that occur during embryonic development and to healing of wounds in adult tissue, e.g., inflammation, angiogenesis (development of new blood supply) and tissue remodeling (Balkwill et al., 2005; Coussens and Werb, 2002). During wound healing, inflammatory cells are recruited to sites of injury to eliminate potential bacterial infection as well as to facilitate healing by providing growth factors and proteases that are essential to the process. In so doing, a new blood supply is also formed that further helps the tissue heal. When 'healing' is complete, inflammation resolves and the tissue returns to its former state. Several of these parameters are conserved during tumor development; however, instead of initiating a 'healing' response, inflammatory cells provide growth-promoting factors that help tumors grow. These observations are significant in light of the fact that individuals suffering from chronic inflammatory diseases harbor a greatly increased risk for cancer development in tissues infiltrated by activated leukocytes (de Visser et al., 2006), and indicate that by identifying molecular mediators regulating onset, activation and maintenance of inflammation in the neoplastic microenvironment, we will reveal regulatory events/molecules that can be effectively targeted with anti-cancer therapeutics.

The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated (Balkwill and Mantovani, 2001). We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. To address these issues, we have taken several approaches to investigate mechanisms involved in: *i.* induction and maintenance of chronic inflammatory microenvironments in premalignant tissues, and *ii.* role of leukocytes and their soluble mediators as regulators of cancer development.

By studying mouse models of skin, lung and breast cancer development, we have recently appreciated that adaptive leukocytes differentially regulate of innate immune cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas chronic inflammation is B cell–dependent during skin carcinogenesis, during mammary carcinogenesis, T cells appear to play more of a dominant role. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells.

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22nd Aspen Cancer Conference: Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention and Cancer Therapy, Aspen CO, USA

Immune Modulation of Epithelial Cancer Development

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The Coussens lab focuses on the role of inflammatory cells and leukocyte proteases as critical regulators of skin, lung and breast cancer development. Our research is based upon the premise that, in addition to intrinsic changes occurring within neoplastic cells, e.g., activation of oncogenes and inactivation of tumor suppressor genes, extrinsic factors, e.g., inflammation, extracellular matrix (ECM) remodeling and angiogenesis also regulate critical properties of tumor evolution.

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Inflammation and Cancer: Organ-specific Regulation of Cancer Development

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2007 Timberline Symposium on: 3D tissue Biology: Human Stem Cells, Cancer and the Microenvironment. Portland, OR.

CD4+ T CELLS FUNCTIONALLY CONTRIBUTE TO MAMMARY TUMOR PROGRESSION.

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During tumor development, the environment in which young 'tumor' cells develop determines their ability to progress to the malignant state. Recent clinical and experimental evidence supports a critical link between inflammation and the development and progression of breast cancer. In neoplastic human breast tissue, the presence of B and T lymphocytes increases during disease progression. In the present study, we assessed the functional significance of infiltrating adaptive immune cells during development of mammary adenocarcinomas by intercrossing the MMTV-PymT mammary carcinoma model with mice deficient for B and T lymphocyte-deficient mice ($RAG1^{-/-}$), only B lymphocytes ($JH^{-/-}$) and or $CD4^+$ and/or $CD8^+$ T lymphocytes. We found that while loss of B or T lymphocytes did not alter latency of primary tumor development, tumor burden or tumor histopathology, the formation of pulmonary metastasis (>80%) was significantly diminished in a $CD4^+$ T lymphocyte-dependent manner. Genetic elimination of $CD4^+$ T cells correlated with deceased numbers of circulating $PymT^+$ tumor cells and diminished presence of M2 'polarized' macrophages present in primary tumors. Using an organotypic 3-dimensional co-culture model with primary $PymT^+$ mammary epithelial cells (MECs) and tumor associated macrophages and $CD4^+$ T cells, we revealed that tumor-associated $CD4^+$ T cells regulate macrophage behavior/phenotype (polarization), that in turn regulate malignant and invasive behaviors of MECs in an interleukin (IL) 4-dependent manner. Together, these data indicate that chronic activation of $CD4^+$ T lymphocytes regulates production of type 2 inflammatory cytokines such as IL-4, that in-turn elicits pro-tumor (as opposed to cytotoxic) bioactivities in macrophages enhancing malignant and metastatic programming of neoplastic mammary tissue.

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And

2008 Keystone Symposium, '*Inflammation, Microenvironment and Cancer'*, Snowbird Resort in Snowbird, Utah, USA

Inflammation and Cancer: Organ-specific Regulation of Cancer Development

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The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have recently appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas chronic inflammation of premalignant skin neoplasms is B cell–dependent, during mammary carcinogenesis, T cells appear to play more of a dominant role in regulating pro-tumor and pro-metastatic properties of myeloid cells. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and thoughts on how these properties can be harnessed for effective anticancer therapeutics.

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CD4+ T cells regulate macrophage phenotype and functionally contribute to mammary tumor progression.

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During tumor development, the environment in which young 'tumor' cells develop determines their ability to progress to the malignant state. Recent clinical and experimental evidence supports a critical link between inflammation and the development and progression of breast cancer. Historically, leukocytes found in and around developing tumors were thought to represent an attempt of the host to eradicate transformed neoplastic cells; however, recent epidemiologic and experimental evidence supports a *promoting* role for some immune cell types during cancer development. In neoplastic human breast tissue, B and T lymphocyte presence increases during progression from pre-malignant *in situ* to malignant disease. In the present study, we assessed the functional significance of infiltrating adaptive immune cells during development of mammary adenocarcinomas by intercrossing the MMTV-PymT mouse mammary carcinoma model with B and T lymphocyte-deficient mice ($RAG1^{-/-}$), B cell-deficient mice ($JH^{-/-}$) and mice deficient for either or both $CD4^+$ and/or $CD8^+$ T cells. We found that while loss of B and T lymphocytes did not alter latency of primary tumor development, tumor burden or tumor histopathology, pulmonary metastasis (>80%) and total metastatic tumor burden were significantly ($p < 0.01$) diminished in a $CD4^+$ T lymphocyte-dependent manner. Genetic elimination of $CD4^+$ T cells phenocopied the MMTV-PymT/$RAG1^{-/-}$ phenotype, as well as correlated with deceased numbers of circulating $PymT^+$ tumor cells and diminished presence of M2 'polarized' macrophages present in primary tumors. Using an organotypic 3-dimensional co-culture model with primary $PymT^+$ mammary epithelial cells (MECs) and naïve or tumor associated macrophages and $CD4^+$ T cells, we revealed that tumor-associated $CD4^+$ T cells regulate macrophage behavior/phenotype (polarization), that in turn regulate malignant and invasive behaviors of MECs in an interleukin (IL) 4-dependent manner. Together, these data indicate that chronic activation of $CD4^+$ T lymphocytes regulates production of type 2 inflammatory cytokines such as IL-4, that in-turn elicits pro-tumor (as opposed to cytotoxic) bioactivities in macrophages that then enhance malignant and metastatic programming of neoplastic mammary tissue.

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